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The Inhibition of RNA-Polymerase II-Mediated Expression by the Non-Structural Protein NSs of the Oropouche Virus and Establishing an Oropouche Virus Minireplicon System

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Abbreviations

Abbreviations

A	
Aa	Amino acid
Ab	Antibody
Amp	Ampicillin
AP-1	Activator protein 1 *(*ATF-2/c-Jun heterodimer)
Arbovirus	Arthropod-borne virus
B	
BHK-21	Baby hamster kidney fibroblast-21
bp	Base pair
BSA	Bovine serum albumin
BSR-T7	BHK-21 cell line stably expressing the T7 RNA polymerase under the control of a CMV promoter
BUNV	Bunyamwera virus
C	
CA	Constitutively active
cAMP	Cyclic adenosinmonophosphate
CBP	CREB-binding protein
CCD	Charge-coupled device
cDNA	Complementary DNA
CMV	Cytomegalovirus
CpG	Cytosin phosphodiester bond guanine
CREB	cAMP response element binding
D	
DMEM	Dulbecco`s modified eagle medium
DNA	Deoxyribonucleic acid

dNTP	Deoxynucleotide triphosphate
ds	Double stranded
E	
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular regulated kinase
F	
FCS	Fetal calf serum
FF	Firefly
FFLuc	Firefly luciferase
G	
G418	Geneticin 418
I	
IFN	Interferon
IFNAR	Interferon-α/-β receptor
Ig	Immune globulin
IgG	Immune globulin G
IL	Interleukin
IPS1	Interferon-α-promoter-stimulator 1
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulatory factor
ISG	Interferon-stimulated gene
ISRE	Interferon-stimulated regulatory element
J	
JAK	Janus kinase

K

kb	Kilo base
kD	Kilo dalton

L

LACV	La Crosse virus
LAR II	Luciferase assay reagent II
LB	Luria-Bertani broth
LUC	Luciferase

M

MAPK	Mitogen-activated protein kinase
MAVS	Mitochondrial anti-viral signaling molecule
MDA-5	Melanoma differentiation associated gene 5
MEKK	MAPK/ERK kinase kinase
mRNA	Messenger RNA
MyD88	Myeloid differentiation primary response gene

N

N	Nucleoprotein
NCBI	National Center for Biotechnology Information
NF-κB	Nuclear factor κB
Nod	Nucleotide oligomerization domain
NS	Non-structural
NSm	Non-structural protein of the M-Segment
NSs	Non-structural protein of the S-Segment
nt	Nucleotide
NTR	Non-translated region
nm	Nanometer

O

ORF	Open reading frame
OROV	Oropouche virus

P

PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline salt
PCR	Polymerase chain reaction
pDCs	Plasmacytoid dendritic cells
PFA	Para formaldehyde
PKA	Protein kinase A
PKR	Protein kinase R
PLB	Passive lysis buffer
Poly (I:C)	Poly (Inositol:Cytosin)
PRDs	Positive regulatory domains
PRRs	Pattern recognition receptors
PS	Penicillin-streptomycin

R

REN	Renilla
RENLuc	Renilla luciferase
RIG-I	Retinoic acid inducible gene I
RIP-1	Receptor-interacting protein 1
RNA	Ribonucleic acid
RNAP II	Cellular RNA polymerase II
RNP	Ribonucleoprotein-(Complex)
RT	Reverse transcriptase
RVFV	Rift Valley fever virus

S

s	Second

SOCS1	Suppressor of cytokine signaling 1
ss	Single stranded
STAT	Signal transducer and activator of transcription
SV40	Simian virus 40
T	
TANK	TRAF family members-associated NF-κB activator
Taq	Thermos aquaticus
TBK-1	TANK-binding kinase 1
TBP	TATA-box binding protein
TE	Tris/EDTA-Buffer
TF	Transcription factor
TFIIH	Transcription factor II H
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF	Tumor necrosis factor (TNF) receptor associated factor
Tris	Tris (-hydroxymethyl)-amino methane
Tyk	Tyrosine kinase
V	
Vero cells	African green monkey cells
VSV	Vesicular stomatitis virus
W	
wt	Wild type

1 Introduction

1.1 The Oropouche Virus

The Oropouche virus (OROV) is an emerging virus that causes a very severe and acute febrile dengue-like illness called the Oropouche fever. This RNA virus is responsible for multiple and massive epidemic outbreaks in South America involving hundreds of thousands of infected people (Vasconcelos et al. 2009). OROV is an arthropod-borne virus, which is transmitted from one person to the next by the biting midge, *Culicoides paraensis*. Despite its current relevance very little is known about the virulence factors of OROV.

1.1.1 Epidemiology

ORO fever has grown to become the second most common arthropod-borne viral disease in Brazil, surpassed only by dengue (Figueiredo 2007). The Oropouche virus (OROV) was first isolated in 1955 from a febrile forest worker in Trinidad (Anderson et al. 1961). Only shortly after its first isolation, in 1961, the first urban outbreak of ORO fever was reported in Belem, Brazil with approximately 11,000 infected persons (Pinheiro et al. 1981). Since then, the virus caused 30 large, explosive outbreaks in cities and villages across Brazil and in neighboring South American countries, with some of the outbreaks involving up to 110,000 infected persons. The last reported epidemic was in 2006 in Brazil's Amazon region and was apparently limited by the cessation of rainfall (Vasconcelos et al. 2009).

Although the OROV currently causes its epidemics every 1 to 7 years in South American countries (with reports from Brazil, Panama, Peru and Trinidad), it has to be noted that the OROV epidemics have been associated with deforestation, colonization, unplanned urbanization and climate change (Vasconcelos et al. 2001). The possibility therefore exists that the arthropod vector of OROV, *Culicoides paraensis* increases its area of circulation. A close relative to this OROV vector, *Culicoides imicola*, which transmits the Bluetongue virus (family *Reoviridae*, genus Orbivirus) has already, for the first time, spread to northern Europe due to climate change and an increase in temperature (Elliott 2009). It is therefore probable that the OROV eventually confronts Middle and North America with epidemic outbursts of the ORO fever. Studies by the Tropical Medicine Foundation of Amazonas State have even shown a

constantly increasing area of circulation of the OROV and they have noted an increasing epidemic potential of the sickness (Mourãão et al. 2009).

1.1.2 Clinical Aspects

1.1.2.1 **Symptoms**

The Oropouche virus causes the Oropouche fever. An Oropouche infection manifests itself in the form of an acute febrile episode, which can be very debilitating. This episode is accompanied by headache (99.1 %), chills (59.3 %), muscle pain (46.9 %), dizziness (39.8 %), photophobia (38.1 %), nausea/vomiting (36.3 %), and joint pain (21.2 %) (Vasconcelos et al. 2009).

These symptoms usually reoccur a few days after the first febrile episode; they are then however less severe. Some patients also develop aseptic meningitis. Patients however usually fully recover without any apparent after effects, even in most serious cases. The incubation period of the virus ranges from four to eight days. Most infections are symptomatic with a ratio of symptomatic to asymptomatic of 2:1 (Pinheiro et al. 1982). Diagnosis of the Oropouche fever occurs mainly serologically (Saeed et al. 2001)

1.1.2.2 **Therapy**

Currently, there is no causal therapy. Only symptomatic interventions such as oral analgesic and anti-inflammatory agents are used. The OROV is resistant against the nucleoside analog Ribavirin (Livonesi et al. 2006). OROV is insensitive to IFN after infection is established (Livonesi et al. 2007); therefore, IFN works only as a prophylaxis. There is also no vaccination available.

1.1.3 Classification

The OROV belongs to the Simbu serogroup of the genus Orthobunyavirus, which is one of the five genera within the *Bunyaviridae* family.

The large virus family *Bunyaviridae* is one of the most extensive virus families with over 350 members. All of its members are RNA viruses that have a tri-segmented, single stranded genome of negative polarity. The members of this family are divided into 5 genera based on

their serological and biochemical properties. Four of the five genera, namely the Orthobunya-, Phlebo-, Nairo-, and the Hantavirus, contain vertebrate-infecting viruses, whereas the genus Tospovirus contains only plant-infecting viruses. Irrespective of their serological and biochemical similarities, the bunyaviruses show diversity in terms of their hosts and vectors, and in their genome coding and replication strategies (Elliott 1997). With relevance to this thesis, the relationship between the Oropouche virus and the La Crosse, Rift Valley fever and Bunyamwera virus should be noted.

Genus	Serogroup	Human pathogenic	Animal pathogenic	Plant pathogenic
Orthobunya virus	California virus	La Crosse virus (= encephalitis)		
	Simbu group	Oropouche virus (= fever)		
	Bunyamwera virus		Bunyamwera virus	
Phlebovirus	Phlebotomus fever virus	Rift Valley fever virus (= encephalitis)	Rift Valley fever virus	
Nairovirus	CCHF* virus	CCHF* virus (= hemorrhagic fever)	CCHF* virus	
Hantavirus	Hantaanvirus	Hantaanvirus (= hemorrhagic fever)		
Tospovirus				Tomato-Spotted-Wilt virus

Table 1: **The five genera of the *Bunyaviridae* family**. Examples of pathogenic viruses, the sicknesses they cause in humans and their preferred host species are stated under every serogroup. The genus Orthobunyavirus has three serogroups. * = Crimean Congo hemorrhagic fever

1.1.3.1 **Structure and Genome**

The Oropouche virus is an enveloped virus of approximately 100 nm in diameter. Just like the other members of the *Bunyaviridae* family, the Oropouche virus has a tri-segmented, single-stranded RNA genome (Elliott and Weber 2009).

The largest segment of the OROV genome is called the L-, the medium-sized segment the M- and the smallest segment the S segment. All of these RNA segments are of negative polarity. Each of the segments is encapsidated with viral nucleocapsid proteins. This encapsidation forms the ribonucleocapsid (RNP) complex and it is solely this complex that serves as the template for the viral polymerase during transcription and replication (Elliott 1997).

The three genome segments encode four structural proteins that make up the viral particle and two Non-structural proteins, one of which (NSs protein) is known to be a major virulence factor of Orthobunya- and Phleboviruses amongst the *Bunyaviridae*.

The L (large) RNA segment encodes the L protein, the RNA-dependent RNA polymerase (approx. 248 kD; 2250 amino acids; BeAn19991 strain). OROV has to take along this polymerase in its viral particles because of its encoding strategy, which requires the transcription of its negative sense RNA into positive sense RNA. Approximately 25 copies of the L protein are associated with the ribonucleocapsid complex of every virus particle (Aquino et al. 2003).

The M (medium) segment encodes the viral surface proteins, glycoprotein Gn (approx. 103 kD; 939 amino acids) and Gc (approx. 32 kD; 290 amino acids), which project from the virus' surface by approximately 10 nm and are involved in the attachment of the virus to the host cell. In between both the Gn and the Gc genes, is the NSm gene which produces the 19 kD (175 amino acid) NSm protein . Little is known about this protein. The molecular masses are calculated based on the amino acid sequence of the OROV prototype strain TRVL 9760 (Wang et al. 2001).

The S (small), 754-nucleotide segment encodes the 231-amino acid structural nucleoprotein, also known as the N-protein. There are approximately 2100 N-protein units per complete virus particle and they encapsidate each of the genome RNA segments. The N-gene contains another gene, which encodes a smaller 91-amino acid non-structural protein, the NSs protein. The NSs open reading frame exhibits a +1 frameshift with respect to the N open reading

frame. Therefore translation of the S segment transcript leads to the production of both N and NSs protein by alternative initiation of translation at the AUG codons of the open reading frames of N and NSs (Elliott and McGregor 1989), (see **Figure 2**). The nucleotide and amino acid numbers of the S segment refer to those of the prototype OROV strain, TRVL 9760, isolated in Trinidad (Saeed et al. 2000).

Phylogenetic analysis of the different OROV strains showed that three genotypes could be distinguished. To genotype I belong the prototype strain from Trinidad and most of the strains isolated in Brazil, genotype II contains the six Peruvian strains that were isolated between 1992 and 1998, and two strains isolated from western Brazil in 1991. Genotype III contains four strains isolated in 1989 in Panama (Saeed et al. 2000). A new, fourth genotype has also recently been described which include Brazilian strains from the Amazonas state (Vasconcelos et al. 2011)

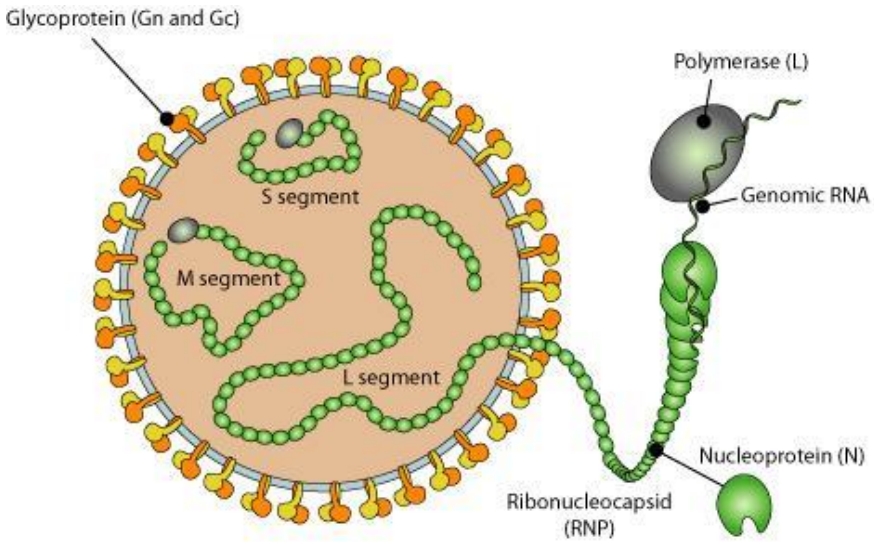

Figure 1. The prototype structure of the *Bunyaviridae* with their tri-segmented genome. The genomic RNA segments are encapsidated with the nucleoprotein. The figure also shows the association between the L protein and the RNP complex. (Picture from ViralZone, viralzone.expasy.org)

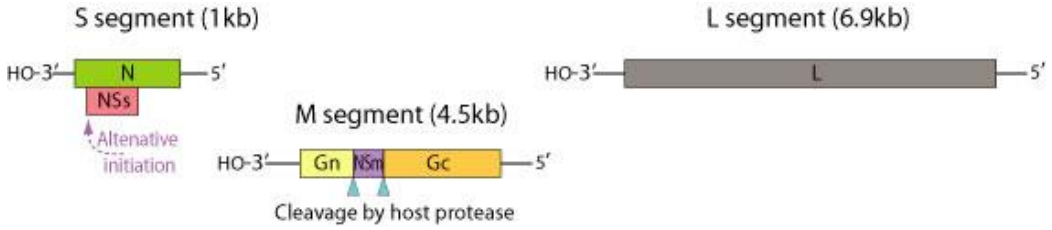

Figure 2. Scheme of the genomic organization of Orthobunyaviruses. (Picture from ViralZone, viralzone.expasy.org)

1.1.3.2 **Transcription and Replication**

The entire replication cycle of the *Bunyaviridae* has not yet been fully understood. However, it is known that the OROV adheres to an unknown receptor on the surface of host cells using its glycoproteins, Gn and Gc. The OROV particles are taken up by receptor-mediated endocytosis. After uncoating by low pH-dependent membrane fusion and entry into the cytoplasm, the viral polymerase (L-protein, a RNA-dependent RNA polymerase) starts transcription of the genomic negative sense RNA (Elliott and Weber 2009).

Firstly, the L protein, which has an endonuclease enzyme property, cuts off a few nucleotides including the cap structure at the 5' end of cellular mRNAs present in the cytoplasm (cap-snatching). The L protein then uses these oligonucleotides as its primer, pairing it to each of the three viral genome segments. The L protein then begins attaching nucleotides to the primer, producing viral mRNA which contains 12 to 18 nucleotides of cellular origin and which is capped at its 5' end. The viral mRNA synthesis then terminates at 50 to 150 nt before the end of the genomic template. The 3' end is not polyadenylated (Elliott and Weber 2009).

This naked viral mRNA is then translated by cellular ribosomes. Once the N (nucleocapsid) protein has reached a critical level, it interacts with the L protein and the RNA synthesis switches to replication mode. This time, RNA synthesis is primer-independent and a full-length, exact complementary copy of the genome in positive sense orientation is produced (the antigenome). During synthesis of this antigenome, it is encapsidated by the pool of N proteins prepared beforehand. The resulting ribonucleocapsid complex serves as a template for the synthesis of progeny negative-sense genomes that are also encapsidated (Elliott and Weber 2009).

Assembly of the viral glycoproteins and the ribonucleocapsid complexes as wells as budding of virions takes place in the Golgi apparatus of the host cell which leads to the release of viral progeny from the infected host cell.

1.2 The Innate Immune Response to Viruses: Type I Interferons

1.2.1 Interferon Types and their Molecular Properties

IFN proteins are commonly grouped into three types based on their unique, respective receptors and their different roles in immune processes. The type I Interferons are also called the viral IFNs and consist of IFN-α, IFN-β and IFN-ω, -ε , -τ, -δ and -κ. Type II IFN is also known as the immune IFN (IFN-γ) and is involved in allergic response, in tumor control and in host defense against intracellular pathogens (Weerd and Nguyen 2012). The type III IFNs have only been recently described and comprise IFN-*γ*1, -*γ*2 and -*γ*3 (Kotenko et al. 2002).

Type I and type III IFNs are induced by viral infection and type II IFN by mitogenic and antigenic stimuli. Most cell types are able to produce Type I IFNs in response to viruses. Only a few special cells are able to produce type II IFN, such as activated Natural Killer cells, CD4 Th1 cells and CD8 cytotoxic suppressor cells (Bach et al. 1997).

The type I interferon system constitutes a very powerful first line of defense against viral infections (Randall and Goodbourn 2008). IFN-*α* and -*β* genes are promptly induced in a cell in response to viral infection. These IFNs activate a signal-transduction pathway that trigger the transcription of a diverse set of genes that, in total, establish an antiviral response in the infected cell itself and in neighboring ones. The efficiency with which a virus can antagonize this cellular IFN system is an important factor of its pathogenicity. Recent research carried out by the Virology Institute, Göttingen (Keisers) showed that cells infected with the Oropouche virus are prevented from producing sufficient IFN amounts.

Humans have a large number of type I IFN genes: 14 IFN-α genes, 1 IFN-β and 1 IFN-ω gene (Pestka et al. 2004) They all lack introns and are clustered on the short arm of chromosome 9. The IFN-α genes have been studied in mice and, based on these studies, can be divided into two groups: the immediate-early response gene, IFN-α4, which is expressed very quickly and

the second group of IFN-α genes which are expressed much more slowly. IFN-α gene products seem to function as monomers and IFN-β and IFN-γ as homodimers (Bach et al. 1997). IFN-β was the first of the IFNs to be purified and characterized. It is best understood. IFN-β has a wide range of important biological effects on the human immune system (Huber and Farrar 2011).

1.2.2 Interferon Induction

Type I IFNs are produced and secreted by cells in response to viral infection, long before the adaptive immune system can produce specific antibodies. These IFNs (-α/-β) activate a number of so-called IFN-stimulated genes (ISG) in the infected cell as well as in uninfected surrounding cells. A number of these expressed ISGs produce proteins that directly or indirectly antagonize viral multiplication. Three phases of the interferon system can be distinguished. Firstly, the host cell has to recognize the virus and start its intracellular signaling processes that lead to the transcription and translation of the IFN genes (IFN induction). Secondly, the secreted IFNs initiate a signaling cascade, which leads to the expression of antiviral proteins (IFN signaling), and lastly, the synthesized IFN-induced antiviral proteins establish an antiviral state.

Recognition: Four major pattern recognition receptor families are known to play a big role in host cells recognition of invading pathogens: Toll-like receptors (TLRs), cytosolic RIG-I-like receptors (RLR), Nod-like receptors and C-type lectins.

TLRs are expressed in cells of the specific immune system. They are found on the cell surface as well as on the membranes of some intracellular organelles, which are normally (in a non-infected state) free of RNA. The binding of certain non-self ssRNA (TLR7, TLR8), dsRNA (TLR3) and CpG DNA (TLR9) to these receptors trigger certain recognition pathways that subsequently lead to the acquisition of an antiviral state (Kawai and Akira 2010).

The two Nod-like receptor family members Nod1 (Chamaillard et al. 2003) and Nod2 (Girardin et al. 2003) have been identified to play more of a role in the recognition of the intracellular bacterial components that are produced during degradation or synthesis of bacterial peptidoglycan.

As TLRs are expressed only in specific immune cells, they cannot account for the anti-viral reaction, for example, in epithelial cells. Cytosolic RLRs and Nod-like receptors that are expressed in most cell types are responsible for the anti-viral reaction in other invaded cells. The detection of cytoplasmic viral RNA is largely carried out by RLR family members such as MDA5 and RIG-I. They recognize so-called PAMPs, which are conserved pathogen-associated molecular patterns. The binding of viral RNA to RIG-I/MDA5 starts complex pathways that end in the activation of the three distinct transcriptional factors IRF-3, the heterodimers NF-κB and ATF-2/c-Jun (AP-1) that are required for the production of IFN and inflammatory cytokines (Hornung et al. 2006, Kato et al. 2011). RIG-I has the ability to bind to the uncapped 5' triphosphorylated RNA end of a single stranded viral genome, as does another intracellular protein called PKR (Hornung et al. 2006). This cellular recognition is practical, because many viruses cannot cap their own RNA, as human cells do. In addition to the capping of RNA, human cells distinguish self from non-self RNA by methylating the ribose sugar molecule at position 2'-O at the 5'-end of the mRNA. MDA5 recognizes this and is activated by viral RNA that lacks this cell-typical signature (Züst et al. 2011).

Once the C-terminal RNA helicase domain of RIG-I and MDA5 have come into contact with viral RNA, the RLRs undergo a conformational change. This change allows them to associate with the adaptor protein MAVS located on the outer mitochondrion membrane. This complex is responsible for the downstream activation NF-κB and IRF-3/IRF-7.

The activated RIG-I/MDA5 and MAVS adaptor protein activate TBK1 and IKKε that together with the aid of the protein TRAF3 phosphorylate IRF-3 and IRF-7 that are constitutively present in the host cells cytosol (Fitzgerald et al. 2003). Phosphorylated IRF-3 either homodimerizes or heterodimerizes with IRF-7 and translocates to the cell nucleus where it serves as a transcriptional factor (Honda et al. 2006).

NF-κB is permanently bound to inhibiting proteins in the cytosol of unstimulated cells called IκB. This inhibiting protein prevents the nuclear translocation of the transcription factor NF-κB. The complex of activated RIG-I/MDA5, the MAVS adaptor protein together with the IKK family members TBK1 and IKKε however phosphorylate this inhibiting protein. Phosphorylated IκB dissociates itself from NF-κB, is marked with ubiquitin and it is subsequently proteolytically

degraded (Karin and Ben-Neriah 2000). In contrast to IRF-3/-7 activation TRAF6 is the required protein (Fitzgerald et al. 2003).

ATF-2/c-Jun (AP-1) is also activated during viral infection by a complex cascade of reactions (Du et al. 1993).

IFN-β gene transcription and translation is initiated, when the transcription factors AP-1, NF-κB and IRF-3, all of which are activated by viral infections, bind to the nucleosome-free (open) enhancer of the IFN-β gene which is -107 to -47 bp upstream of the transcriptional starting point (Panne et al. 2007). All three factors have to interact with each other and the enhancer region for the IFN-β gene to be transcribed (Maniatis et al. 1998). IRF-3 is constitutively expressed in cells. Upon viral infection, the signaling processes result in the phosphorylation of its C-terminal regulatory domain, permitting its homodimerization and translocation to the cell nucleus, where it binds to CBP/p300 (Takahasi et al. 2010). This enables the phosphorylated IRF-3 to bind to the IFN-β enhanceosome. Of the three transcriptional factors activated upon viral infection, IRF-3 appears to be the most important. It alone has the ability to induce the IFN-β gene expression in the absence of the other two transcriptional factors, provided its concentration is high enough (Hiscott et al. 1999).

Two nucleosomes cover the TATA-box and the transcription start site of the IFN- β gene. Enzymes first have to acetylate the histones in the nucleosome, which then move away and give general transcription factors like the TATA-box binding protein access to the region (Lomvardas and Thanos 2001). The enhanceosome (enhancer + IRF-3/7 + NF-κB + AP-1) then recruits the RNA polymerase II and activates IFN-β mRNA synthesis (Panne et al. 2007). The mRNA is translated into the IFN-β protein in the cytoplasm.

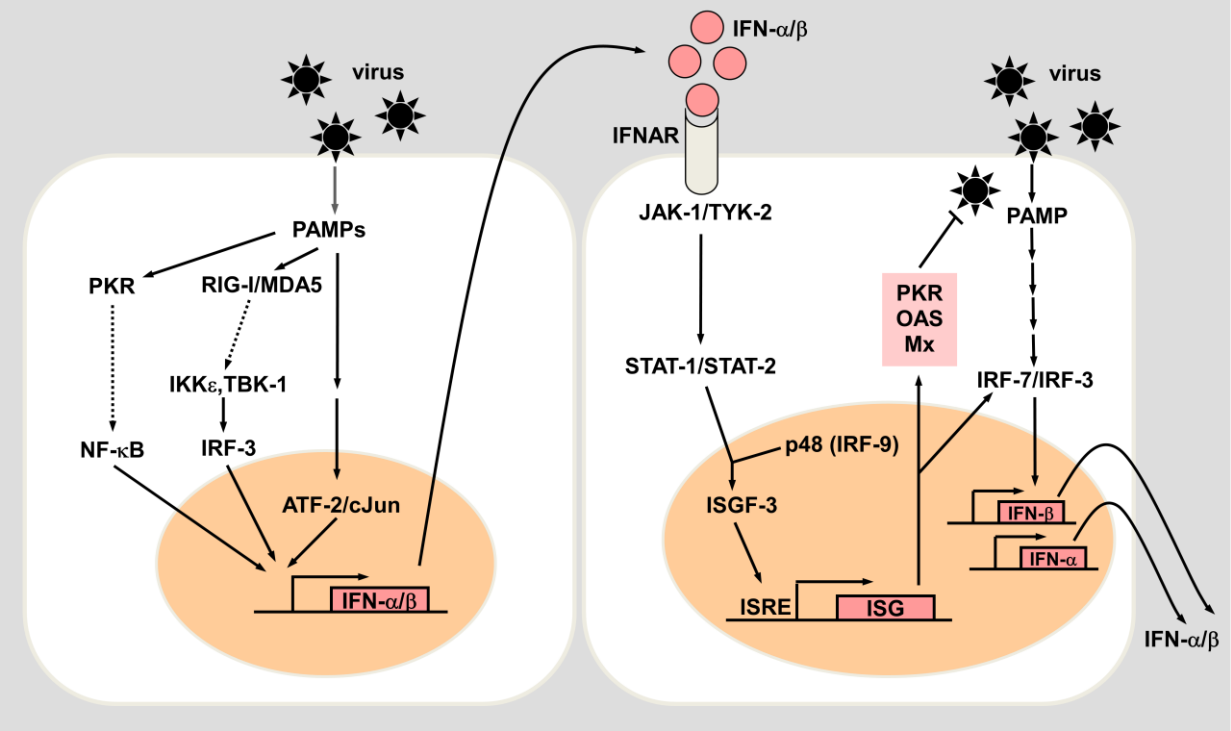

Figure 3. Overview of interferon induction and interferon effects. Three critical transcription factors, IRF-3, NF-κB and AP1 (ATF-2/cJun) are activated after viral recognition and they bind to the enhancer of the IFN-β gene, enabling its expression. (Figure from Dr. rer. nat. M. Spiegel, Virology Institute, Göttingen)

1.2.3 Interferon Signaling

The different IFN-α/–β subtypes all bind to a common type I IFN receptor (IFNAR). These receptors are ubiquitously and constitutively expressed (de Weerd et al. 2007). All the studied IFN-α and –β isoforms bind to this IFNAR, however with different affinities. IFNAR is composed of two type I heterodimeric, transmembrane subunits, called IFNAR-1 and IFNAR-2. The janus kinases JAK-1 and TYK-2 are permanently associated with the IFNAR. The N-terminus of signal transducer and activator of transcription 2, shortly called STAT 2, associates itself with the cytoplasmic part of IFNAR-2, before cytokine activation. Upon IFN binding the two IFNAR subunits co-ligate and activate the JAK-STAT signaling pathway: JAK proteins phosphorylate the tyrosine residues within the cytoplasmic domain of IFNAR1/-2 and in the cytoplasmic STAT-2 protein, which is associated with IFNAR-2. Phosphorylated STAT-2 recruits STAT-1 from the cytoplasm to form a heterodimer. Phosphorylated STAT-1 and STAT-2 associate with another protein called IRF-9. . This complex is now called the interferon-stimulated gene factor-3, ISGF-3. This ISGF-3 activates the majority of the interferon-stimulated genes, ISGs,

after translocation to the cell nucleus where it binds to the interferon-stimulated response elements (ISRE) found in the promoters of ISGs (Huber and Farrar 2011, Schindler et al. 2007), see **Figure 3**.

Over 300 ISGs exist that have antiviral, antiproliferative, and immunomodulatory functions. These include enzymes, diverse transcription factors, surface glycoproteins, cytokines, chemokines and others with unknown function. IRF-7 is an example of one of the transcription factors produced that is capable of activating many other members of the IFN gene family. Its production increases the cascade reactions once viral products have been recognized by the cell. Once IRF-7 is phosphorylated in infected cells, it becomes active (Marie et al. 1998). The major known proteins with direct antiviral activity are the Mx GTPases, PKR and the oligoadenylate synthetases. Mx GTPases target viral nucleocapsids, thereby inhibiting RNA synthesis. PKR that is activated by dsRNA and phosphorylates the cellular initiation factor eIF-2α, thereby inhibiting translation of both viral and cellular mRNAs. The oligoadenylate synthetases activate the RNase L in the cytoplasm, which then degrades RNA in the cytoplasm (Samuel 2001).

Furthermore, type I IFN induces in a positive feedback loop Nod2 (Kim et al. 2011) and RIG-1 (Hu et al. 2011). This increases immune surveillance in the local microenvironment establishing a higher cellular resistance to infection.

Type I IFN bridges the gap between the innate (which mainly produces it) and the adaptive immune system. Plasmacytoid dendritic cells produce large amounts of type I IFN. Apart from its function in the innate immune system, type I IFN activates $CD8^+$ T cells and supports cross-presentation. Likewise type I IFN promotes the differentiation of $CD4^+$ T-cells into T helper cells type 1 (Th1), whereas the differentiation into Th2 and Th17 is restricted (Huber and Farrar 2011). IFN-β also up-regulates MHC type I molecules on the dendritic cell membrane (Inácio et al. 2012); it promotes dendritic cell maturation (Santini et al. 2000) and activation (Luft T et al. 1998).

1.2.4 Bunyaviridae counter-actions to Interferon

Bunyaviruses possess a tri-segmented genome, which means that a viral particle has three putative RIG-1-activating 5' triphosphate ends in its ssRNA genome. To counterbalance this, Orthobunyaviruses produce a very potent antagonist of the IFN system, the so-called NSs protein (Weber et al. 2002). The so far characterized NSs proteins of the Orthobunyaviruses Bunyamwera (Thomas et al. 2004) and La Crosse (Blakqori et al. 2007) and of the Phlebovirus RVFV (Billecocq et al. 2004) that have been studied until now are known to inhibit RNA polymerase II-mediated cellular transcription thereby preventing IFN synthesis. These respective NSs proteins however all have a different inhibition mechanism.

1.3 Aim of the study

This thesis aims on the one hand at characterizing Oropouche virus' major virulence factor, the so-called Non-structural (NSs) protein. On the other hand, it aims at setting up a minireplicon system for the Oropouche virus that will eventually allow for the targeted manipulation of its genome, permitting an insight into the viruses' replication and transcription processes, and shedding some light on virus-host cell interactions. Understanding these processes could pave the way for developing therapeutic interventions against this currently untreatable disease.

2 Material and Methods

2.1 Material

2.1.1 Chemicals

The following is a list of the chemicals that were used during experimental work with their respective acquisition companies.

Chemicals	Company
10 x Transcription buffer	Roche Diagnostics GmbH, Mannheim, Germany
Ammonium acetate	
Distilled Water for PCR (DNase-/RNase-free)	Invitrogen Corporation, Carlsbad, California, U. S. A.
Dithiothreitol (DTT)	Serva GmbH, Heidelberg, Germany
Dulbecco's PBS [10X]	c.c.pro GmbH, Oberdorla, Germany
Ethanol	Carl Roth, Karlsruhe, Germany
FCS	c.c.pro GmbH, Oberdorla, Germany
FluorSave™ Reagent	Calbiochem, San Diego, California, U. S. A.
Isopropanol	Merck KgaA, Darmstadt, Germany
L-Glutamin [200 mM]	c.c.pro GmbH, Oberdorla, Germany
Paraformaldehyde	Sigma, Munich, Germany
Passive Lysis Buffer [5x]	Promega Corporation, WI, U. S. A.
Purified Bovine Serum Albumin (BSA) [10 mg/ml]	New England BioLabs Inc., Ipswich, MA, U. S. A.
Random-Hexamer-Primer	GE Healthcare, Freiburg, Germany
Triton X-100	Serva GmbH, Heidelberg, Germany
Trypan Blue (0.4 %)	Sigma-Aldrich, St. Louis, U. S. A.

2.1.2 Buffers and Solutions

The following is a list of the buffers and solutions that were used during experimental work and their composition.

Buffers and Solutions	Constitution
0.5% Triton X-100	250 µl in 50 ml 1 x PBS

1 % FCS/PBS	1 µl FCS in 99 µl 1 x PBS
1 x PLB	10 µl PLB [5 x] in 40 µl distilled water
3 % Para formaldehyde	3 g Paraformaldehyde in PBS
BSA [1 mg/ml]	10 µl BSA [10 mg/ml] in 90 µl distilled water
Dulbecco's PBS [10X]	2 g KCl, 2 g KH_2PO_4, 80 g NaCl, 5,76 g N_2HPO_4, ad 1 l H_2O
Dulbecco's PBS [1X]	100 ml PBS [10X] ad 900 ml H_2O
DMEM	500 ml DMEM, 50 ml FCS, 100 U/ml Penicillin, 100 µg/ml Streptomycin, and 526,6 mg/l L-Glutamin
LB-Agar with Ampicillin	10 g Bacto Tryptone, 5 g Bacto Yeast Extract, 10 g NaCl, 1 ml Ampicillin [100 µg/ml], 15 g Bacto Agar, ad 1 l H_2O
LB-Medium with Ampicillin	10 g Bacto Tryptone, 5 g Bacto Yeast Extract, 10 g NaCl, 1 ml Ampicillin [100 µg/ml], ad 1 l H_2O

2.1.3 Kits and Sets

The following is a list of the kits and sets that were used during experimental work with their respective acquisition companies.

Kits and Sets	**Company**
DNA-*free*	Ambion Inc., Texas, U. S. A.
Dual-Luciferase Reporter Assay	Promega Corporation, Madison, U. S. A.
FuGENE 6 Transfection Reagent	Roche Diagnostics GmbH, Mannheim, Germany
Lipofectamine Transfection Reagent 2000	Invitrogen Corporation, Carlsbad, California, U. S. A.
NucleoBond PC100 Plasmid Purification	Macherey-Nagel GmbH & Co. KG, Düren, Germany
Quant-iT RiboGreen RNA Assay	Invitrogen Corporation, Carlsbad, California, U. S. A.
rNTP Set [Each 20 µmol]	Roche Diagnostics GmbH, Germany
RNA Cap Structure Analog (7mG(5')ppp(5')G Sodium salt)	New England BioLabs Inc., Ipswich, MA, U. S. A.
Transmesenger Transfection Reagent	QIAGEN GmbH, Hilden, Germany

2.1.4 Antibiotics

The following is a list of the antibiotics that were used during experimental work with their respective acquisition companies.

Antibiotics	Company
Ampicillin	Serva, Heidelberg, Germany
G418 (Geneticin)	c.c.pro GmbH, Oberdorla, Germany
Penicillin Streptomycin (PS)	c.c.pro GmbH, Oberdorla, Germany

2.1.5 Cell lines

The following are the cell lines that were used. Their respective origins, source and culture media are also stated.

Cell lines	Origin tissue (Organism)	Acquired from	Culture medium
293	Embryonal kidney (Human)	Microbix Biosystems Incorporated, Ontario, Canada	DMEM supplemented with 10 % FCS
BSR-T7	Kidney (Hamster)	Prof. Dr. K. Conzelmann, Ludwig-Maximilians-University, Munich, Germany	DMEM supplemented with 10 % FCS + G418 (120 µl in 12 ml DMEM)
Vero E6	Kidney epithelium (African green monkey)	Gerhard Dobler Microbiology Institute of the Armed Forces, Munich, Germany	DMEM supplemented with 10 % FCS

2.1.6 Bacteria

The following are the Bacteria that were used during experimental work with their respective acquisition companies.

Bacteria	Genotype	Company
XL1-Blue	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F′ proAB lacIqZΔM15 Tn10 (Tetr)][c]	Stratagene, Santa Clara, California, U. S. A.

2.1.7 Media

The following is the list of the media that were used during experimental work with their respective acquisition companies.

Media	Company
DMEM	c.c.pro GmbH, Oberdorla, Germany
Opti-MEM I Medium	Invitrogen Corporation, Carlsbad, California, U. S. A.

2.1.8 Plasmids

The following is a list of the plasmids that were used, their respective description and their source. Every listed plasmid has an ampicillin resistance gene. This ampicillin resistance is for selecting the E. coli that have incorporated the plasmids during transformation (see 2.2.3 Transformation of Plasmid-DNA).

Plasmids	Description	Source
p125-luc	The firefly luciferase gene expression of this plasmid correlates with the IFN-β promoter activation	Takashi Fujita, The Tokyo Metropolitan Institute of Medical Sciences, Tokyo, Japan
p55A2-luc	This reporter plasmid is controlled by a NF-κB-responsive promoter and expresses firefly luciferase	Takashi Fujita, The Tokyo Metropolitan Institute of Medical Sciences, Tokyo, Japan
p55C1B-luc	This reporter plasmid is controlled by an IRF-3-responsive promoter and expresses firefly luciferase	Takashi Fujita, The Tokyo Metropolitan Institute of Medical Sciences, Tokyo, Japan
pAP-1-luc	This 5.7 kb reporter plasmid is controlled by an AP-1-responsive promoter and expresses firefly luciferase	Stratagene, Santa Clara, California, U. S. A.
pFC-MEKK	Controlled by the constitutively active CMV-Promoter, expressing MEKK	Stratagene, Santa Clara, California, U. S. A.
pGL3-FF-luc	Control plasmid for the expression of firefly	Promega, Mannheim,

	luciferase under the control of the constitutively active SV40 promoter	Germany
pI.18	Empty eukaryotic expression vector. Carries the constitutively active hCMV IE-promoter and the hCMV intron A followed by a multiple cloning site for the insertion of cDNA and a hCMV polyA signal	Jim Robertson, National Institute for Biological Standards and Control, Hertfordshire, United Kingdom
pI.18-FLAG-ΔMx	Expression plasmid for N-terminal FLAG-tagged and truncated Mx protein	Prof. Dr. Friedemann Weber, Universitätsklinikum, Freiburg, Germany
pI.18-OROV-NSs-Flag	Expression plasmid for C-terminal FLAG tagged NSs of OROV	Virology Institute, Göttingen, Germany
pI.18-RNSs-ZF5	Expression plasmid for C-terminal FLAG tagged NSs of RVFV strain ZH548	Virology Institute, Göttingen, Germany
pI.18-RVFV-NSs Clone 13	Expression plasmid NSs of RVFV strain clone 13	Virology Institute, Göttingen, Germany
pI.18-RVFV-NSs-Z1	Expression plasmid NSs of RVFV strain ZH548	Virology Institute, Göttingen, Germany
pIRF-3(5D)	Expression plasmid for a constitutively active phosphomimetic form of IRF-3	John Hiscott, McGill University, Montreal, Canada
pRL-SV40	Expression plasmid for renilla luciferase Expression is driven by the constitutively active SV40 promoter	Promega, Mannheim, Germany
pT7-ribo-LACV-vMpro-vRL	This LACV minigenome plasmid has a T7 polymerase promoter and contains the renilla luciferase gene in genomic orientation. The 3’ and 5’ ends are from NTRs of the LACV M segment in genomic orientation	Prof. Dr. Friedemann Weber, Universitätsklinikum, Freiburg, Germany
pT7-riboSM2-cMpro cRL	This OROV minigenome plasmid has a T7 polymerase promoter and contains the renilla luciferase gene in antigenomic	Virology Institute, Göttingen, Germany

	orientation. The 3’ and 5’ ends are from NTRs of the OROV M segment in antigenomic orientation	
pT7-riboSM2-orov-vMpro-vRL	This OROV minigenome plasmid has a T7 polymerase promoter and contains the renilla luciferase gene in genomic orientation. The 3’ and 5’ ends are from NTRs of the OROV M segment in genomic orientation	Virology Institute, Göttingen, Germany
pTM1-FFLuc	This reporter plasmid has a T7 polymerase promoter and an EMCV-IRES followed by the firefly luciferase cDNA and the T7 terminator	Prof. Dr. Friedemann Weber, Universitätsklinikum, Freiburg, Germany
pTM1-OROV-cL	This constitutively active plasmid is controlled by a T7 polymerase promoter and expresses the OROV L protein	Virology Institute, Göttingen, Germany
pTM1-OROV-cSmut	This constitutively active plasmid is controlled by a T7 polymerase promoter and expresses the OROV N protein. The NSs protein is mutated to a dysfunctional form	Virology Institute, Göttingen, Germany
pTM1-LACV-L	This constitutively active plasmid is controlled by a T7 polymerase promoter and expresses the LACV L protein	Prof. Dr. Friedemann Weber, Universitätsklinikum, Freiburg, Germany
pTM1-LACV-N	This constitutively active plasmid is controlled by a T7 polymerase promoter and expresses the LACV N protein	Prof. Dr. Friedemann Weber, Universitätsklinikum, Freiburg, Germany
pUC19	pUC19 is a standard high-copy cloning vector for *E. coli* recombinants.	Virology Institute, Göttingen, Germany

2.1.9 Primary Antibodies

The following are the primary antibodies that were used.

Primary Antibodies	Description	Target	Company
ANTI-FLAG Polyclonal	Polyclonal rabbit IgG antibody	DYKDDDDK sequence in fusion proteins	Sigma-Aldrich, St. Louis, Missouri, U. S. A.
Monoclonal ANTI-FLAG M2	IgG1 monoclonal antibody, isolated from murine ascites fluid	DYKDDDDK sequence in fusion proteins	Sigma-Aldrich, St. Louis, Missouri, U. S. A.
Pol II (N-20)	Polyclonal rabbit IgG antibody	N-terminus of RNA polymerase II	Santa Cruz Biotechnology Inc., Dallas, Texas, U. S. A.
RNA polymerase II H14 antibody	Monoclonal IgM antibody from murine ascites	Phospho-serine 5 in the heptapeptide repeat of the C-terminal domain of the RNA polymerase II	Covance, San Diego, California, U. S. A.
RNA polymerase II H5 antibody	Monoclonal IgM antibody from murine ascites	Phospho-serine 2 in the heptapeptide repeat of the C-terminal domain of the RNA polymerase II	Covance, San Diego, California, U. S. A.

2.1.10 Secondary Antibodies

The following are the secondary antibodies that were used.

Secondary Antibodies	Description	Target	Company
Cy 3-conjugated Anti-mouse antibody	IgG Anti-mouse antibody developed in Donkey	Fc part of mouse antibodies	Dianova, Hamburg, Germany
FITC-conjugated Anti-rabbit antibody	IgG Anti-rabbit antibody developed in Goat	Fc part of rabbit antibodies	Sigma, St. Louis, U. S. A.

2.1.11 Proteins, Enzymes, and Enzyme Inhibitors

The following is a list of the proteins, enzymes and the enzyme inhibitors that were used with their respective acquisition companies.

Enzymes, Protein Inhibitors and Other Proteins	Company
Tumor Necrosis Factor Alpha	Sigma, St. Louis, U. S. A.
T7 RNA polymerase [20 U/µl]	Roche Diagnostics GmbH, Mannheim, Germany
Trypsin/EDTA Solution	Biochrom AG, Berlin, Germany
rRNasin RNase Inhibitor [40 U/µl]	Promega, Mannheim, Germany
RiboLock RNase Inhibitor	Fermemtas GmbH, St. Leon-Rot, Germany

2.1.12 Devices and Equipment

The following is a list of the devices and the equipment that were used with their respective acquisition companies

Devices and Equipment	Company
Autoklav Typ Tecnoclav 50	Tecnomara, Zürich, Switzerland
Bacteria Incubator	Haraeus Instruments GmbH, Hannover, Germany
Basic Glass Utilities	Schütt, Göttingen, Germany
Big pipette tips (2 ml, 5 ml, 10 ml, 25 ml)	Hirschmann Laborgeräte GmbH & Co. KG, Eberstadt, Germany
Cell counting chamber	Hycor Biomedical Inc., California, U. S. A.
Cell culture flasks with filter (75 cm^2, 25 cm^2)	Greiner Bio-One GmbH, Frickenhausen, Germany
Cell culture plates (6 well, 12 well)	Greiner Bio-One GmbH, Frickenhausen, Germany
Centrifuge	Eppendorf AG, Hamburg, Germany
Centrifuge (Megafuge 1.0R)	Thermo Scientific, Rockford, U. S. A.
Chemidoc XRS System	Bio Rad Laboratories GmbH, Munich, Germany
Falcon tubes (15 ml, 50 ml)	Sarstedt AG & Co, Nümbrecht, Germany
Filter paper	Whatman GmbH, Dassel, Germany
FLUOstar OPTIMA Reader	BMG LABTECH GmbH, Offenburg, Germany
FLUOTRAC 600 96-well plate	Greiner Bio-One GmbH, Frickenhausen, Germany
Freezer (-140°C)	Thermo Scientific, Rockford, U. S. A.
Freezer (-20 °C)	Liebherr GmbH, Ochsenhausen, Germany

Freezer (-80 °C)	Thermo Scientific, Rockford, U. S. A.
Fridge (5°C)	Liebherr GmbH, Ochsenhausen, Germany
Gel electrophoresis apparatus	Invitrogen Corporation, Carlsbad, California, U. S. A.
Ice machine	Ziegra, Isernhagen, Germany
Incubator	Heraeus Sepatech GmbH, Osterode, Germany
Incubator bath	Köttermann GmbH & Co KG, Uetze/Hänigsen, Germany
Laboratory paper cloth (20,5 cm 20 cm)	Kimberly- Clark Europe Limited, Kings Hill, UK
Laser Scanning Spectral Confocal Microscope Leica TCS SP2	Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany
Latex gloves	Mikroflex Corporation, Vienna, Austria
LUMITRAC 600 96-well plate	Greiner Bio-One GmbH, Frickenhausen, Germany
Microwave	AFK Deutschland GmbH, Hamburg, Germany
Multiple Channel pipette 50 – 200 µl	Eppendorf AG, Hamburg, Germany
Multitex cleaning serviettes	Zellstoff-Vertriebs-GmbH & Co. KG, Troisdorf, Germany
NanoDrop ND-1000 Spectrophotometer	PEQLAB Biotechnologie GmbH, Erlangen, Germany
Nikon Eclipse TS100 microscope	Nikon Instruments Europe B.V, Amstelveen, Netherlands
Nitrile gloves	GE Healthcare Europe NV, Brussels, Belgium
Para film	American National Can, Chicago, U. S. A.
PCR chamber	G&P Kunststofftechnik, Kassel, Germany
PCR tubes (0.2 ml)	Biozym, Scientific GmbH, Oldendorf, Germany
PCR tubes (0.2 ml)	Greiner Bio-One GmbH, Frickenhausen, Germany
Petri dishes 10 cm diameter	Greiner Bio-One GmbH, Frickenhausen, Germany
Pipette tips with filter (0.1 - 10 µl, 1.0 - 100 µl, 101 - 1000 µl)	Starlab GmbH, Ahrensberg, Germany
Pipette tips without Filter (0.1 - 10 µl, 1.0 - 100 µl, 101 - 1000 µl)	Starlab GmbH, Ahrensberg, Germany
Pipettes	Hirschmann Laborgeräte GmbH & Co. KG, Eberstadt, Germany
Safe Lock Tubes (0.5 ml, 1.5 ml, 2 ml)	Eppendorf AG, Hamburg, Germany

Scale	Satorius, Göttingen, Germany
Sterile chamber	Heraeus Sepatech GmbH, Osterode, Germany
Thermo cycler	Biometra GmbH, Göttingen, Germany
Thermo mixer	Eppendorf AG, Hamburg, Germany
Vortex	Bender & Hobein AG, Zürich, Switzerland
Waste bags (300 mm x 200 mm)	Lab Logistic Group GmbH, Meckenheim, Germany

2.1.13 Software

The following is a list of the software that was used with their respective acquisition trademark.

Software	Company
Adobe Photoshop	Adobe, San Jose, California, U. S. A.
FLUOstar OPTIMA Version 1.32 R2	BMG LABTECH GmbH, Offenburg, Germany
Microsoft Office 2007	Microsoft Inc., Redmond, Washington, U. S. A.
ND-1000 V 3.3.0 one channel	PEQLAB Biotechnologie GmbH, Erlangen, Germany
NIS Elements Br	Nikon, Düsseldorf, Germany
Windows XP	Microsoft Inc., Redmond, Washington, U. S. A.

2.2 Methods

2.2.1 Maintaining Cell Lines

General methodical principles:

To avoid overgrowth of cells and cell damage, the cells are split approximately two times in a week. The different cell lines are stored in T75 (75 cm^2) cell culture bottles at 37 °C and in a 5 % CO_2 atmosphere.

Procedure:

The 293 and Vero E6 cell lines are each cultivated in 12 ml of DMEM and the BSR-T7 cell line in 12 ml of DMEM that contains 120 µl of G418. The splitting process is carried out as follows: The culture medium is removed from the culture bottles and the adherent cells are washed with 4 – 5 ml of PBS to get rid of residual FCS (which contains a potent inhibitor of trypsin). 1

ml Trypsin is added and incubated for 2 min at RT. Light hitting of the bottle on its side helps to detach the cells. 4 ml of the cell growth medium DMEM (ready-to-use) is added to the cell suspension. Approximately 0.5 ml of this suspension is refilled into the culture bottle and the rest discarded. The bottle is filled with 11.5 ml medium to attain its initial 12 ml (equivalent to a cell split ratio of 1:10). In the case of the BSR-T7 cell line, 120 µl G418 is then added. The cultivation bottles are then placed into the cell incubator.

G418 is an aminoglycoside antibiotic that interferes with 80 S ribosomes, blocking protein synthesis. It is used for the selection of eukaryotic cells that have been stably transfected with neomycin resistance genes (e. g. the BSR-T7 cell line).

2.2.2 Determining Cell Number

General methodical principles:

It is of utter importance that experiments remain comparable. To achieve this, the cells are counted before an experiment is carried out on them.

Procedure:

10 µl of the cell suspension (made up of 1 ml trypsin and 4 ml DMEM) that was collected during the splitting process (see 2.2.1, Maintaining Cell Lines) is pipetted into a 1.5 ml tube.

90 µl of Trypan Blue is then added to make the cells more visible under the microscope. 10 µl of this new suspension is pipetted into a Neubauer cell-counting chamber. The cells in approximately 3 squares are counted and the average calculated. The cell number is then estimated using the following formula: Average x 10^5 = Number of cells per 1 ml of cell suspension.

2.2.3 Transformation of Plasmid-DNA

General methodical principles:

Plasmid-DNA being used during experiments somehow has to be multiplied. E. coli, if made competent, has the ability to incorporate DNA that is in its immediate environment. In nature, this process is aimed at, for e. g. transferring antibiotic resistance between bacteria. These competent E. coli replicate the plasmid-DNA that they incorporate. Each plasmid being used has a gene that induces resistance to the antibiotic, ampicillin. Therefore, the bacteria that

contain enough of this plasmid and are replicating it efficiently are resistant to ampicillin. Placing the bacteria now on a LB-ampicillin-agar plate selects only these resistant bacteria.

Ready-to-use competent bacteria were used for all transformation procedures

Procedure:

The competent E. coli strain XL1 blue stored at -80°C is left to slowly warm up by placing the tube containing the cells on ice. 1 µl of plasmid-DNA is then added directly to the XL1 blue bacteria and shortly stirred. The bacteria are then immediately placed back on ice and left for 30 min. Subsequently, the bacteria are streaked on a LB-ampicillin-agar plate and incubated over night at 37 °C.

2.2.4 Plasmid-DNA Purification

General methodical principles:

A colony of resistant bacteria is picked from the LB-ampicillin-agar plate and is used for inoculation of 5 ml LB-ampicillin medium. The tube is then placed in an incubator shaker at 150 rpm and 37 °C for 6 hours. 1 ml of this mixture is then pipetted into an Erlenmeyer flask containing 50 ml of the LB-ampicillin medium. The flask is then placed in the incubator shaker for 16 hours at 150 rpm and 37 °C.

The plasmid-DNA now has to be extracted from the bacteria and purified of other bacterial components (see Plasmid-DNA Purification, 2.2.4). For this, the NucleoBond PC100 system is used. This system employs an alkaline/SDS lysis procedure to lyse the bacteria releasing both chromosomal and plasmid DNA. They are both however in a denatured form under such alkaline conditions. A potassium acetate buffer is then added to this denatured lysate. This precipitates chromosomal DNA and other cellular compounds. Plasmid-DNA, which remains in solution, can revert to its native super coiled structure when the solution is neutralized by the potassium acetate buffer. A NucleoBond column with equilibration buffer is used in the system to bind the plasmid-DNA to an anion-exchange resin. After repeated washing of the column, the bound plasmid-DNA is eluted under high-salt conditions. The eluted DNA is then precipitated by adding 2-Propanol. The DNA pellet is then washed twice with 70 % Ethanol to remove the salt used for elution. After briefly drying the DNA pellet, it is then dissolved in

distilled water. The amount of plasmid now just has to be determined (see 2.2.5, Quantification of Plasmid-DNA using NanoDrop).

Procedure:

The purification of plasmid-DNA is carried out according to the manual provided by the manufacturer (Macherey-Nagel).

2.2.5 Quantification of Plasmid-DNA using UV spectrometry

General methodical principles:

It is necessary that the concentration of the purified plasmid-DNA be determined. The NanoDrop® ND-1000 Spectrophotometer is used for analyzing the DNA samples. A pulsed xenon flash lamp is the light source that produces ultraviolet light of 260 nm wavelength and a linear CCD array is used to detect the light after passing through the sample. Nitrogenous bases (purine and pyrimidine) in DNA absorb this ultraviolet light. Bases have their peak absorption at the wavelength 260 nm. The amount of light absorbed when passing through the sample is therefore directly proportional to the concentration of DNA in the sample.

Procedure:

A 1.5 µl plasmid-DNA sample is pipetted onto the end of a fiber optic cable (the receiving fiber). A second fiber optic cable (the source fiber) is then brought into contact with the liquid sample causing the liquid to bridge the gap between both fiber optic ends and the measurement is carried out.

2.2.6 Transfection of eukaryotic cells with Plasmid-DNA

General methodical principles:

For introduction of plasmid-DNA into eukaryotic cells Lipofectamine 2000 (Invitrogen) is used. This is a formulation of cationic and neutral lipids that complexes with nucleic acids : the charged side of the cationic lipid interacts with the negatively charged DNA, with its neutral lipid chain sticking outwards away from the DNA. The neutral lipids also in the mixture associate with this neutral tail of the cationic lipids, thus allowing fusion with the lipid cell membrane. This fusion allows the complexed plasmid-DNA, which is negatively charged, to be transferred over the cellular membrane and into the cell.

Procedure:

The 293 cell line is chosen, because this cell line has one of the highest transfection efficiencies when using Lipofectamine 2000 for transfection. 24 hours before transfection, the cells are seeded at 1 x 10^5 cells per well (see 2.2.2) in a 12-well plate in 1 ml DMEM (ready-to-use). The cells are grown for 24 hours under their normal growth conditions (37°C and 5 % CO_2). 2 µl of the Lipofectamine Reagent is diluted in 50 µl of Opti-MEM I Medium for each well. The mixture is incubated at room temperature (15 – 25°C) for 5 min. During this time, the different corresponding are diluted in 55 µl of the Opti-MEM I medium and vortexed for a few seconds. Both mixtures (plasmid and the reagent) are added together and incubated for 20 min at RT to allow formation of DNA-lipid complexes. 100 µl of this mixture is then added to the well containing the cells and DMEM (ready-to-use). The plate is gently rocked back and forth. The cells are subsequently incubated under their normal growth conditions (37°C and 5 % CO_2) for 24 h. They are then lysed and the firefly and renilla luciferase activities are measured (see 2.2.10).

Experiments concerning the transcription factors IRF-3, NF-κB and AP-1: In the test series of reporter assays 0.25 µg of the reporter plasmids is transfected per well, unless otherwise stated (see Results). An equivalent amount of pUC19 (0.25 µg) is transfected into corresponding wells and serves as the control.

0.5 µg of the plasmids expressing either the OROV NSs protein (pI.18-OROV-NSs-FLAG) or that of the RVFV (pI.18-RVFV-NSs-Z1) is transfected per well. In corresponding wells, the equivalent amount of the empty vector (pI.18) is transfected.

12.5 ng of pRL-SV40 that serves as the control is transfected per well, unless otherwise stated (See Results).

2.2.7 Generating RNA from Plasmid-DNA by *in vitro* Transcription

General methodical principles:

Here, plasmid-DNA is transcribed into RNA by *in vitro* transcription using the T7 RNA polymerase. The DNA plasmids that were transcribed here into RNA all have the T7 promoter (pTM1-FFLuc and pT7-riboSM2-cMpro cRL).

A mixture containing the T7 RNA polymerase and the four different triphosphate nucleotides is incubated. The polymerase binds specifically to the T7 promoter of the plasmid DNA and catalyzes the synthesis of complement RNA in the 5´→ 3´ direction based on the DNA template. This polymerase is one of very active bacteriophage polymerases that easily transcribes plasmid-DNA in a cell-free milieu to RNA. Unlike the eukaryotes RNA polymerase II that requires a number of cofactors for promoter binding, this T7 RNA polymerase is uncomplicated and can synthesize RNA without these additional factors. Once the RNA created *in vitro* is introduced into a cell, the cell begins its translation into protein. Therefore, here, the translation process of the cell is analyzed independent of the preceding transcription.

Procedure:

The *in vitro* transcription is carried out as follows: distilled, DNase- and RNase-free water is added to 2 µg of plasmid-DNA such that the total volume is 10 µl. 10 µl of 10 x transcription buffer, 5 µl of 1 mg/ml BSA, 5 µl of 0.1 M DTT, 5 µl RNase inhibitor, 16 µl 2.5 mM rNTP (2.5 mM per rNTP), 47 µl distilled (DNase/RNase-free) water and 2 µl T7 RNA polymerase are all added. This mixture of 100 µl is incubated at 37 °C for 1.5 h. After this incubation period, 11 µl of 10 x DNase buffer and 1 µl of Ambions rDNase I (Ambions DNA-*free*™ Set) are added to destroy the DNA template. The mixture is incubated at 37 °C for 30 min. 10 µl of DNase Inactivation Reagent (Ambions DNA-*free* Set) is then added and the mixture is left at room temperature (15 – 25 °C) for 2 min and then mixed shortly. The mixture is centrifuged for 1.5 min at 10,000 g and the supernatant transferred to a new 1.5 ml vial. 6.7 µl of 7.5 M Ammonium acetate is added and then 110 µl Isopropanol to precipitate the newly transcribed RNA. The vial is cooled for at least 15 min at -20 °C and is then centrifuged for 15 min at 12,000 g. The supernant is discarded and the RNA pellet is washed twice with 200 µl 75 % ethanol each time; in between washes, the cup is centrifuged for 10 min at 10,000g. After drying the RNA pellet 50 µl of distilled (RNase-free) water is added to resuspend the RNA. The RNA is quantified using RiboGreen (see Quantification of RNA using RiboGreen, 2.2.8). The RNA solution is then stored at -20 °C.

2.2.8 Quantification of RNA using RiboGreen

General methodical principles:

In determining the concentration of the RNA generated from the *in vitro* transcription of Plasmid-DNA, the Quant-iT RiboGreen RNA Assay is utilized. This RiboGreen RNA quantification relies on a dye that exhibits a large fluorescence enhancement upon binding to RNA (and other nucleic acids). The fluorescence emission is in linear correlation with the amount of RNA. The fluorescence is measured with the FLUOstar OPTIMA reader and the FLUOstar OPTIMA software calculates the concentration of the sample RNA based on comparisons with a ribosomal RNA standard (16S and 23S rRNA from E. coli) in different dilutions. Falsely high concentrations due to RNA degradation are reduced by this system because free nucleotides are not detected.

Procedure:

The quantification procedure is carried out according to the manual of the manufacturer (Invitrogen).

2.2.9 Transfecting Cells with RNA

General methodical principles:

In gene expression, a cellular gene (DNA) is transcribed into RNA, after post-transcriptional modification, this RNA as mRNA is transported out of the cell nucleus into the cytoplasm and ribosomes translate the ribonucleic sequence into an amino acid sequence (protein). So, artificially introducing (transfecting) RNA into a cell skips the transcriptional step and allows direct translation of the encoded protein.

TransMessenger transfection reagent is used for combined DNA and RNA transfection. TransMessenger transfection reagent is based on a lipid formulation and is used with a specific RNA-condensing reagent (enhancer R) and an RNA-condensing buffer (buffer EC-R), all included in the transfection reagent set. In the first step of TransMessenger-RNA complex formation, the RNA is condensed by interaction with enhancer R in a defined buffer system. In the second step, the TransMessenger Transfection Reagent (included in set) is added to the condensed RNA to produce TransMessenger-RNA complexes. The TransMessenger-RNA complexes are then mixed with antibiotics-free medium and added directly to the cells.

Procedure:

The procedure is carried out as follows: 24 hours before transfection, the cells are seeded at 1 x 10^5 cells per well (see Determining Cell Number, 2.2.2) of a 12-well plate in 1 ml DMEM containing antibiotics (PS). The cells are then incubated under their normal growth conditions (37 °C and 5 % CO_2). 2 µl of Enhancer R is diluted in Buffer EC-R. A total of 1 µg of RNA/DNA is added and mixed by vortexing for 10 s. The final volume is always adjusted to 100 µl. The mixture is incubated at room temperature (15 – 25 °C) for 5 min, and is then spun down for a few seconds to collect drops from the top of the tube. 4 µl of TransMessenger Transfection Reagent is added and pipetted up and down 5 times. Samples are incubated for 10 min at room temperature (15 – 25 °C) to allow transfection-complex formation. During this process, growth medium is gently aspired from the wells. The cells are washed once with 2 ml PBS. 300 µl of DMEM without FCS and antibiotics is added to the tube containing the transfection complexes and mixed by pipetting up and down twice. The mixture is then immediately added drop-wise onto the cells and the plate is swirled gently. The cells are then incubated under normal growth conditions for 4 hours. Subsequently, the mixture is removed from the cells, followed by a washing step with 2 ml PBS and then 1 ml DMEM with FCS and antibiotics is added to the cells. The cells are incubated under normal growth conditions for another 24 hours and then lysed and the protein expression was analyzed using the Dual-Luciferase Reporter Assay System (see Dual-Luciferase Reporter Assay 2.2.10).

2.2.10 Dual-Luciferase Reporter Assay

General methodical principles:

This system is used to study the promoter activities using an indirect readout: light emission as a consequence of enzymatic activity. The “dual reporter” refers to the simultaneous expression of two individual enzymes in transfected cells. In this Dual-Luciferase Reporter Assay, the activities of the firefly (*Photinus pyralis*) and the renilla (*Renilla reniformis*) luciferases are sequentially measured. Firefly luciferase (a 61 kDa enzyme) and the renilla luciferase (a 36 kDa enzyme) do not require post-translational modification to emit luminescent signals. Thus, they function as genetic reporters immediately upon translation. The firefly luciferase cDNA is fused with different promoters and expression levels measured

under different conditions (see Results). The renilla luciferase gene is fused with a constitutively active promoter SV40 (pRL-SV40, Promega), and hence mainly served as the control. This control (renilla luciferase activity) is necessary to ensure similar transfection efficiencies between different cell groups such that differences observed in firefly luciferase activity are a consequence of different promoter activities rather than a consequence of different transfection efficiency.

Procedure:

The Dual-Luciferase Reporter Assay is carried out as follows: 24 h post transfection the growth medium is removed from the transfected cells grown in 12 well plates. 100 µl of PBS is gently applied to wash the surface of the cell monolayer. The 12-well plate is then gently swirled to remove detached cells and residual growth medium. This PBS mixture is then completely removed. 100 µl of the 1 x PLB is added to each of the 12 wells and swirled to ensure complete coverage of the cell monolayer. The 12-well plate is intermittently rocked during a 20-minute incubation time at room temperature (15 – 20 °C). The wells are then scraped vigorously with a pipette. 20 µl of this cell lysate is transferred into a well of a Lumitrac 600 96-well plate. 100 µl of ambient temperature LAR II is added to the lysate. The Lumitrac 600 96-well plate is then immediately placed in the FLUOstar OPTIMA reader and the firefly activity is measured. During measurement, the Renilla luciferase substrate (Stop & Glo Reagent) is prepared: for each assay, 2 µl of Stop & Glo Substrate is mixed with 100 µl of the Stop & Glo Buffer. 100 µl of this Stop & Glo Reagent is then added to each well of the 96-well plate once the firefly luciferase measurement is done. The renilla luciferase activity is then measured.

2.2.11 Immunofluorescence

General methodical principles:

Immunofluorescence is the labeling of cell structures, intracellular or surface molecules using specific antibodies. The visualization can either be direct (i. e. the primary antibody is coupled to a fluorescent dye) or indirect. Indirect Immunofluorescence is used throughout this study. It employs two types of antibodies: the primary antibody that is targeted at the antigen of interest and the secondary, dye-coupled antibody that is subsequently added and recognizes the primary antibody. The antibodies of different species have different constant regions and

are therefore only recognized by specific secondary antibodies, for e. g. antibodies generated in a goat against a particular antigen have the goat's constant region. The secondary antibody, that is dye-coupled, has to recognize the primary antibodies' constant region. Then, using a microscope that emits light of a particular wavelength, the dye coupled to the secondary antibody is excited and emits light of a different wavelength. A photo detector or digital camera detects this emission. The information is then compiled in the computer as an image.

Procedure:

The immunofluorescence staining is carried out as follows: The cell line Vero E6 is used, because this cell line tightly adheres to surfaces. Glass cover slips are placed into the wells of a 12-well plate. Vero E6 cells are seeded at 0.5 x 10^5 cells per well of this 12-well plate (see 2.2.2). The cells are incubated under normal growth conditions (37 °C and 5% CO_2) for 24 h. The cells in the wells are then transfected with 1 µg of the relevant plasmids (see 2.2.6) and incubated for 24 h. Fixation of the cells is carried out by transferring the cover slips to a new 12-well plate, which already contained 1 ml of 3% Paraformaldehyde. The cells are then incubated for 10 min at room temperature (15 – 20 °C). The cell monolayer is subsequently washed 3 times with 1 ml of 1 x PBS. The cells are then permeabilized by removing the 1 x PBS, adding 1 ml of 0.5% Triton X-100 and incubating for 5 min/RT. The cells are then washed 3 times with a 1 % FCS in PBS solution. The primary antibodies are prepared by diluting them in an appropriate ratio in 1 % FCS in PBS: the mouse antibodies H5 and H14 were used in the ratio of 1:100 respectively, the mouse monoclonal ANTI-FLAG M2 antibody in the ratio of 1:200, the rabbit ANTI-FLAG polyclonal antibody in the ratio of 1:200, and the rabbit N20 antibody was used in the ratio of 1:50. These were the ratios that were produced the clearest results and were always used throughout the experiments. 1 µl ToPro 3 Iodide is then added to this primary antibody dilution for counterstaining the cell nuclei.

The solution in the wells is removed, and 40 µl of the primary antibody mix is then carefully placed onto the cover slips drop-wise and left for 1 h at RT. Then, the cell monolayer is washed three times with 1 % FCS in PBS. The secondary antibodies are diluted in 1 % FCS in PBS according to their recommended working concentration and placed onto the cover slips: the Cy 3-conjugated anti-mouse antibody was always used in the ratio of 1:100 and the FITC-conjugated anti-rabbit antibody in the ratio of 1:80 for each experiment. 1 µl ToPro 3 Iodide is

again added to the secondary antibody dilution and the mixture is incubated with the cells for 1 h at RT. Subsequently, they are washed three times with 1% FCS/PBS. The cover slips are shortly dipped into a Petri dish containing distilled water and excess water is carefully removed from the cover slip. A drop of mounting medium (Fluosafe, Calbiochem) is placed on an glass slide and the cover slip, with the cell monolayer facing down, is placed into this drop of mounting medium. The slides are then stored at RT until the mounting medium is solidified and afterwards at 4 °C and in darkness until immunofluorescence analysis.

2.2.12 Minireplicon system

Minireplicon systems are powerful tools in analyzing transcription and replication of RNA viruses in the absence of infectious virus. For a few of the members of the Bunyaviridae family minireplicon systems already exist: For RVFV (Lopez et al. 1995), Uukuniemi virus (Flick and Pettersson 2001), Toscana virus (Accardi et al. 2001), Akabane virus (Ogawa et al. 2007), BUNV (Dunn et al. 1995), and for the La Crosse virus (Blakqori et al. 2003).

A minireplicon system consists of a functional viral polymerase and an artificial minigenome, which can be transcribed and replicated by the viral polymerase. The minigenome contains a reporter gene, e.g. the renilla luciferase gene. The reporter gene is flanked by the non-coding regions at the 3’ and the 5’ ends of the wild type virus’ genomic M segment. These non-coding regions of the virus contain the promoters that are recognized by the viral polymerase. This minigenome (renilla gene + viral non-coding regions) has a genomic orientation and the corresponding minigenome plasmid contains the T7 promoter upstream of the minigenome. Once transcribed in the T7 polymerase-expressing cell line BSR-T7, the RNA transcript mimics the viral genome with its antisense (genomic) orientation, which serves as a template for transcription by the viral polymerase. The viral polymerase (L-protein) as well as the viral nucleoprotein (N-protein) is expressed in the cell using helper plasmids. Similar to the minigenome plasmid, expression is T7 polymerase dependent, the cDNAs are provided in sense (antigenomic) orientation and are preceded by an internal ribosomal entry site (IRES). These features allow synthesis of the L- and N-protein in transfected BSR-T7 cells.

The viral N protein can encapsidate the minigenome RNA transcript and the L protein recognizes this as its template, therefore transcribing it to mRNA. Once this mRNA has been

translated into the renilla protein, an increased renilla activity is observed in the Dual-Luciferase Reporter Assay (See 2.2.10).

Principle:

The aim of these experiments is to find the problem that prevented the OROV minigenome that we tried to set up from working properly. In order to achieve this, reporter assays (see above) are carried out.

Firstly, the dysfunctionality of the OROV minireplicon is shown.

Procedure:

The BSR-T7 cell line is chosen here, because this cell line constitutively expresses the T7 polymerase. The plasmids that are used are all T7-promoter controlled and should therefore be constitutively expressed once transfected into the BSR-T7 cells. 24 hours before transfection, the cells are seeded at 5 x 10^4 cells per well (see 2.2.2) in an 18-well plate in 1 ml DMEM (ready-to-use) under their normal growth conditions (37°C and 5 % CO_2). A complete set of experiments is carried out in 6 wells. The other 12 wells permitted a lysis at different times.

3 µl of the FuGENE 6 reagent is diluted in 50 µl of Opti-MEM I Medium for each well. The mixture is then incubated at room temperature (15 – 25°C) for 5 min. During this time, the plasmids (see below) are diluted in 10 µl of the Opti-MEM I medium and vortexed for a few seconds to collect liquid back to the bottom of the vial. Both reagent and plasmid mixture are added together and incubated for 15 min at RT to allow formation of DNA-lipid complexes. 50 µl of this mixture is then added to the well containing the cells. The plate is gently rocked back and forth. The cells are subsequently incubated under their normal growth conditions (37°C and 5 % CO_2). After the first 24 h the cells of the first six wells are lysed, then after 36 h and at 48 h. The firefly and renilla luciferase activities are then measured (see 2.2.10).

The first set of experiments is carried out as shown in the Minireplicon Table 2. Whereby the respective plasmids and their functions have been previously described under “Plasmids” (see 2.1.8).

	1	2	3	4	5	6
pUC19	-	0.3 µg	-	0.3 µg	-	0.3 µg
pTM-LACV-L	0.3 µg	-	0.3 µg	-	-	-
pTM-LACV-N	0.3 µg	0.3 µg	-	-	-	-
pTM1-orov-cSmut	-	-	0.3 µg	0.3 µg	0.3 µg	0.3 µg
pTM1-orov-cL	-	-	-	-	0.3 µg	-
pT7-ribo-LACV-vMpro-vRL	0.3 µg	0.3 µg	-	-	-	-
pT7riboSM2-orov-vMpro-vRL	-	-	0.3 µg	0.3 µg	0.3 µg	0.3 µg
pTM1-FFLuc	0.15 µg	0.15 µg	0.15 µg	0.15 µg	0.15 µg	0.15 µg

Table 2. Minireplicon: The numbers 1 to 6 refer to the wells containing the BSR-T7 cells.The green column is the actual test while the purple column is the control. pTM1-FFLuc was added to all wells and served to prove successful transfection.

The amount of the plasmid is optimized to produce higher renilla activity and the following set of experiments is carried out as shown in the Minireplicon Table 3:

	1	2	3	4	5	6	7	8
pUC19	-	0.6 µg	-	0.6 µg	-	0.6 µg	-	0.6 µg
pTM-LACV-L	0.6 µg	-	0.6 µg	-	-	-	-	-
pTM-LACV-N	0.6 µg	0.6 µg	-	-	-	-	0.6 µg	-
pTM1-orov-cSmut	-	-	0.6 µg	0.6 µg	0.6 µg	0.6 µg	-	-
pTM1-orov-cL	-	-	-	-	0.6 µg	-	0.6 µg	0.6 µg
pT7-ribo-LACV-vMpro-vRL	0.6 µg	0.6 µg	0.6 µg	0.6 µg	-	-	-	-
pT7riboSM2-orov-vMpro-vRL	-	-	-	-	0.6 µg	0.6 µg	0.6 µg	0.6 µg
pTM1-FFLuc	0.1 µg	0.1 µg	0.1 µg	0.1 µg	0.1 µg	0.1 µg	0.1 µg	0.1 µg

Table 3. Minireplicon: The numbers 1 to 8 refer to the wells containing the BSR-T7 cells. The green column is the actual test while the purple column is the control. pTM1-FFLuc was added to all wells and served to prove successful transfection.

2.2.13 Statistical analysis

Statistical analysis of reporter assay data was performed using the two-tailed Welch's t-test for the determination of p values. All reporter assays (see 3.1.1) were repeated 5 times.

3 Results

3.1 Interference of OROV-NSs with IFN-β Promoter Activation

3.1.1 Reporter Assays

Previous experiments have shown that cells infected with the Oropouche virus are prevented from inducing IFN-β (Keisers); however, the mechanism of inhibition is not yet known. The experiments furthermore demonstrated, that transiently expressed OROV-NSs in cells is sufficient to mediate this effect in a virus-free system. The following reporter assays aim at characterizing this phenomenon in more detail: it is known that the positive regulatory elements of the IFN-β promoter bind IRF-3, NF-κB and AP-1 (Panne et al. 2007). We investigated whether OROV-NSs specifically interferes with the activation of one of these transcription factors, whereby the first experiment (see 3.1.1.1) is meant to demonstrate that the activation of the IFN-β promoter with its three different binding sites is indeed inhibited by the OROV-NSs protein. The experiments thereafter then analyze the individual transcription factors for inhibition (see 3.1.1.2 to 3.1.1.4).
293 and BSR-T7 cells were used here for transfections with plasmid DNA or RNA (see 2.2.6 and 2.2.9, respectively).

3.1.1.1 IFN-β Promoter Activation blocked by OROV-NSs

Objective:

Does the OROV-NSs prevent IFN-β promoter activation (and as a consequence IFN-β gene expression)? In other words, if we transfected a plasmid containing the IFN-β gene promoter (fused with a reporter gene) together with the OROV-NSs-expressing plasmid into the same cells and try to activate the promoter, would IFN-β promoter inhibition be seen?

Description:

A constitutively active form of the transcription factor IRF-3 is expressed in the cells of the 293 cell line by means of transfecting them with the plasmid IRF-3(5D). The constitutively active, spontaneously homodimerized IRF-3(5D) binds to the IFN-β promoter of the likewise transfected reporter plasmid p125-luc, inducing the expression of the firefly luciferase (FFLuc) protein (see Figure 4, “Empty Vector”). IRF-3(5D) alone was used to activate the IFN-β

promoter in this experiment, because it is the only factor that has been shown to have the ability to activate the IFN-β promoter all on its own independent of the other two transcription factors NF-κB and AP-1 (Hiscott et al. 1999).

Experiment:

293 cells were transfected with the reporter plasmid p125-luc together with the control plasmid pRL-SV40, which expresses the renilla luciferase (RENLuc) protein (see 2.2.6.). Three sets of experiments were carried out and the results of all three are displayed beside each other on each of the charts (see Figure 4 and Figure 5):

Set 1: The plasmid expressing the OROV-NSs protein (pI.18-OROV-NSs-FLAG) was transfected into the cells of two wells. The IRF-3(5D) plasmid was introduced into the cells of one of these wells. IRF-3(5D) should normally activate the expression of p125-luc-encoded firefly luciferase (see Figure 4, "Empty Vector"). pUC19 was transfected into the other well and served as the unstimulated control.

Set 2: The plasmid pI.18-OROV-NSs-FLAG in the above set was replaced by an equivalent amount of the RVFV-NSs-expressing plasmid pI.18-RVFV-NSs-Z1. The other components remained the same. The plasmid pI.18-RVFV-NSs-Z1 was used instead of pI.18-OROV-NSs-FLAG because it leads to the expression of the NSs protein of the Rift Valley fever virus, which is known to be a very efficient inhibitor of RNA polymerase II-mediated transcription. It is known to inhibit IFN-β transcription even in the presence of the transcription factors IRF-3, NF-κB and AP-1 (Billecocq et al. 2004).

Set 3: The pI.18-OROV-NSs-FLAG in the initial experiment was replaced by an equivalent amount of the empty plasmid, pI.18. This served as the control.

All 6 wells with cells were transfected with p125-luc					
Cells were transfected with pUC19 (unstimulated control)			Cells were transfected with the IRF-3(5D) expression plasmid (stimulation)		
OROV-NSs	RVFV-NSs	pI.18 (Empty Vector)	OROV-NSs	RVFV-NSs	pI.18 (Empty Vector)

Table 2: An overview of the plasmid combinations. The cells of three wells were transfected with pUC19 (unstimulated control) and the cells of the other three wells were transfected with the IRF-3(5D) expression plasmid (stimulation). The OROV-NSs-, the RVFV-NSs-expression plasmids and pI.18 (empty vector) were transfected into the cells of each of the three wells. This was done for the cells of the three wells co-transfected with the IRF-3(5D) expression plasmid and the cells of the three wells co-transfected with pUC19 (control). The blue bars represent firefly luciferase activity of the control cells, that of the cells transfected with the IRF-3(5D) expression plasmid by red bars in Figure 4.

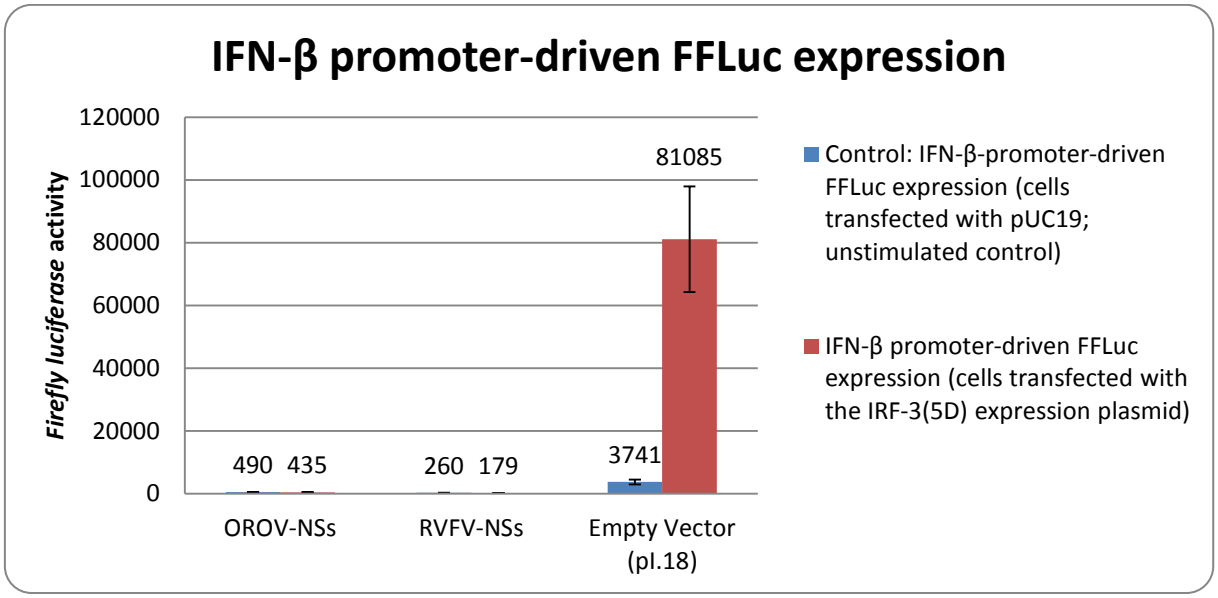

Figure 4. Firefly luciferase activity: effect of the OROV-NSs on the IRF-3-dependent luciferase expression.

Blue: Control (pUC19) **Red: Stimulated (IRF-3(5D))**

Empty Vector (Control): IRF-3(5D) apparently highly and significantly (p = 0.0005) activates the IFN-β promoter and as a result the expression of the firefly luciferase (see "Empty Vector" in **Figure 4**). IRF-3(5D) increases reporter gene expression by factor 22.

RVFV-NSs and OROV-NSs: The major increase by factor 22 that one sees in the control was prevented in the presence of both the RVFV- and OROV-NSs. There was no increment at all to be seen after stimulation (transfection with IRF-3(5D)). The firefly luciferase activity in the cells expressing RVFV-NSs and IRF-3(5D) was even lower compared to unstimulated control cells expressing RVFV-NSs. The luciferase activity of the cells co-transfected with the empty vector pI.18 and the IRF-3(5D) expression plasmid in comparison to that of the cells expressing the RVFV-NSs and IRF-3(5D) was strongly diminished (453 times less, p = 0.0004). This type of potent suppression of IFN-β promoter activation has been described for the RVFV-NSs (Billecocq et al. 2004). In the corresponding sample with OROV-NSs and IRF-3(5D), the firefly luciferase activity likewise was greatly reduced (186 times, p = 0.0004, **Figure 4**, "OROV-NSs") when compared to the control sample (see **Figure 4**, "Empty Vector").

The basal firefly luciferase activity in the presence of either the RVFV- or OROV-NSs was also diminished in comparison to that of the unstimulated control (see **Figure 4**, "Empty Vector"). The firefly luciferase activity was 14 times lower (p =0.0004) in the case of cells transfected

with the RVFV-NSs expression plasmid and 8 times lower (p = 0.0006) when the OROV-NSs expression plasmid was transfected.

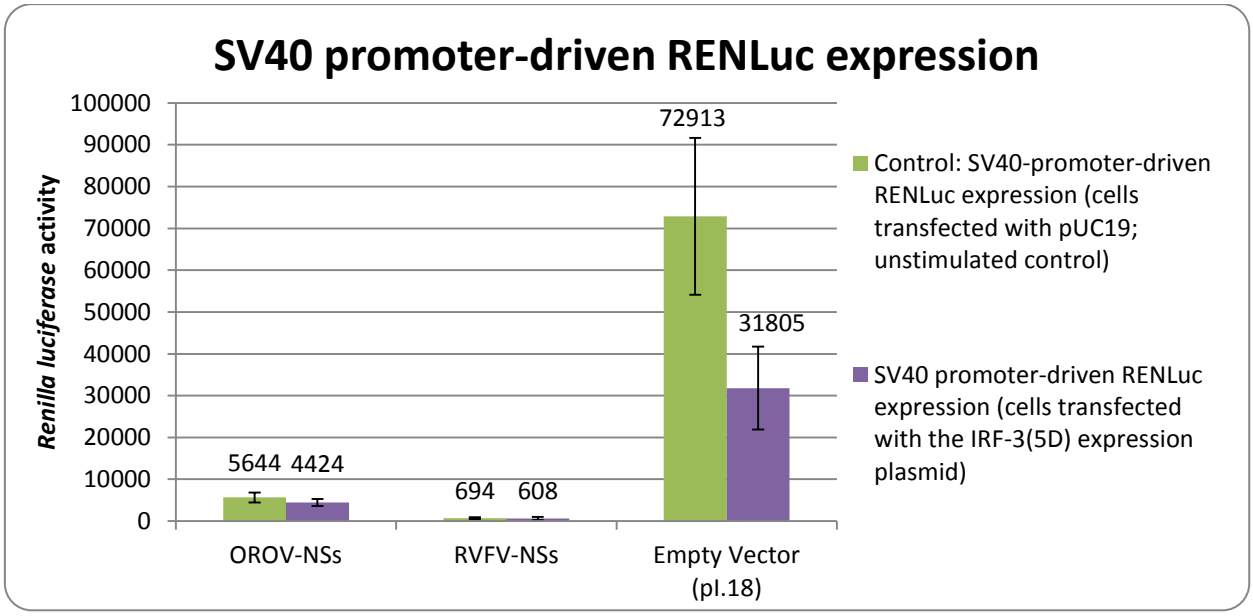

Figure 5. Renilla luciferase activity: internal control of transfection efficiency with pRL-SV40.

Green: Control (pUC19) Purple: Stimulated (IRF-3(5D))

Internal Control: The renilla luciferase gene expression (RENLuc) that is driven by the constitutively active SV40 promoter showed some interesting results. The reporter gene activity was inhibited by OROV-NSs (13 times lower, p = 0.001) and RVFV-NSs (105 times lower, p = 0.001) when compared to the unstimulated, NSs-free control (Figure 5, "Empty Vector"). The RVFV-NSs protein is known to potently inhibit all cellular gene expression at the transcriptional level and produce similar results (Billecocq et al. 2004). The decrease in renilla luciferase activity after transfection of the IRF-3(5D) expression plasmid in the control (Empty Vector) is likely due to the IRF-3(5D)-mediated activation of endogenous IFN-β gene expression, which can lead to inhibition of transcription and translation (Samuel 2001).

3.1.1.2 Transcription Factor IRF-3

Objective:

Does the inhibitory effect of the OROV-NSs lie in inhibiting a late step of IRF-3 activation (= nuclear translocation, interaction with CBP/p300 or the binding to the IRF-3-binding element PRDIII/I)? In other words, if active IRF-3 is artificially generated in a cell (independent of its

intracellular pathway), do we see inhibition of the IRF-3-responsive reporter gene expression when OROV-NSs is present? Previous experiments showed that phosphorylated (active) IRF-3 was present in the cell after stimulation with poly(I:C) or VSV-RNA and in the presence of the OROV-NSs protein (Keisers). This means that the inhibitory effect of the OROV-NSs has to lie downstream of IRF-3 activation (= phosphorylation).

Description:

Just as in the above experiment, the constitutively active form of the transcription factor IRF-3 is expressed in the cells of the 293 cell line by means of transfecting them with the plasmid IRF-3(5D). This constitutively active, artificially homodimerized IRF-3(5D) binds to the promoter of the likewise transfected reporter plasmid p55C1B-luc, inducing the expression of the firefly luciferase protein. The plasmid p55C1B-luc contains an artificial promoter made up of five binding sites for IRF-3, the same one as it is found in the original IFN-β promoter combined with a minimal promoter.

Experiment:

293 cells were transfected with the reporter plasmid p55C1B-luc and with the control plasmid pRL-SV40 (see 2.2.6.). Three sets of experiments were carried out and all three are displayed beside each other on each of the charts (see **Figure 6** and **Figure 7**):

Set 1: The plasmid expressing the OROV-NSs protein (pI.18-OROV-NSs-FLAG) was transfected into the cells of two wells. The IRF-3(5D) plasmid was introduced into the cells of one of these wells and an equivalent amount of pUC19 was transfected into the cells of the other well and served as the unstimulated control.

Set 2: The plasmid pI.18-OROV-NSs-FLAG in the above set was replaced by the equivalent amount of the RVFV-NSs-expressing plasmid pI.18-RVFV-NSs-Z1. The other components all remained the same.

Set 3: The pI.18-OROV-NSs-FLAG in the initial experiment was replaced by the empty plasmid, pI.18. This served as the control.

All 6 wells with cells were transfected with p55C1B-luc					
Cells were transfected with pUC19 (unstimulated control)			Cells were transfected with the IRF-3(5D) expression plasmid (stimulation)		
OROV-NSs	RVFV-NSs	pI.18 (Empty Vector)	OROV-NSs	RVFV-NSs	pI.18 (Empty Vector)

Table 3: An overview of the plasmid combinations. The cells of three wells were transfected with pUC19 (unstimulated control) and the cells of the other three wells were transfected with the IRF-3(5D) expression plasmid (stimulation). The OROV-NSs-, the RVFV-NSs-expression plasmids and pI.18 (empty vector) were transfected into the cells of each of the three wells. This was done for the cells of the three wells co-transfected with the IRF-3(5D) expression plasmid and the cells of the three wells co-transfected with only pUC19 (control). Firefly luciferase activity of the control cells is represented by the blue bars, that of the cells transfected with the IRF-3(5D) expression plasmid by red bars in Figure 6.

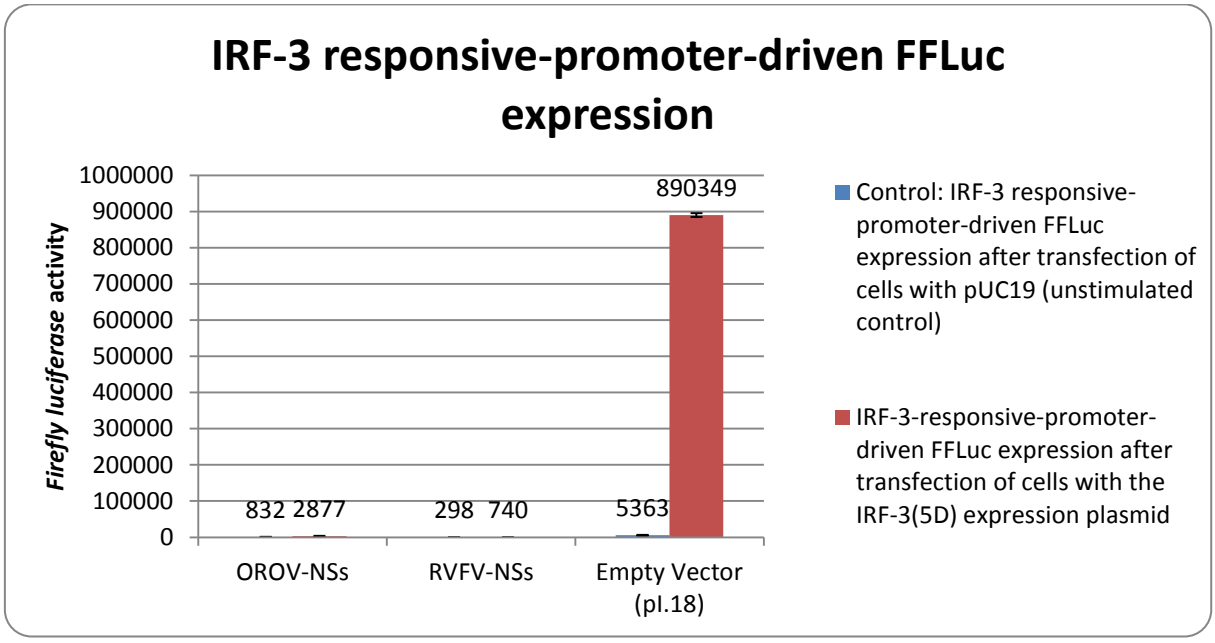

Figure 6. Firefly luciferase activity: effect of the OROV-NSs on the IRF-3-dependent luciferase expression.

Blue: Control (pUC19) **Red: Stimulated (IRF-3(5D))**

Empty Vector (Control): IRF-3(5D) highly and significantly (166 times higher luciferase activity, $p = 3.8^{-10}$) activates the IRF-3 responsive promoter and leads to subsequent firefly luciferase gene expression in the absence of NSs (see "Empty Vector" in **Figure 6**).

RVFV-NSs and OROV-NSs: The results here were very similar to those of the preceding reporter assay. The cells that expressed OROV-NSs that were not transfected with the IRF-3(5D) expression plasmid showed a reduction in luciferase gene expression (by factor 6, p =

0.001) in comparison to the OROV-NSs-free control cells (see **Figure 6**). Similar results were seen for the cells that expressed RVFV-NSs that were not transfected with IRF-3(5D) (activity reduction by factor 18, p = 0.0008). The difference between the cells transfected with IRF-3(5D) and with the OROV-NSs expression plasmid and those of the control transfected with the IRF-3(5D) expression plasmid (without the OROV-NSs expression plasmid) is by the factor of 309 ($p = 3.8^{-10}$), with the RVFV-NSs-expressing cells is the difference by factor 1203 ($p = 3.4^{-10}$).

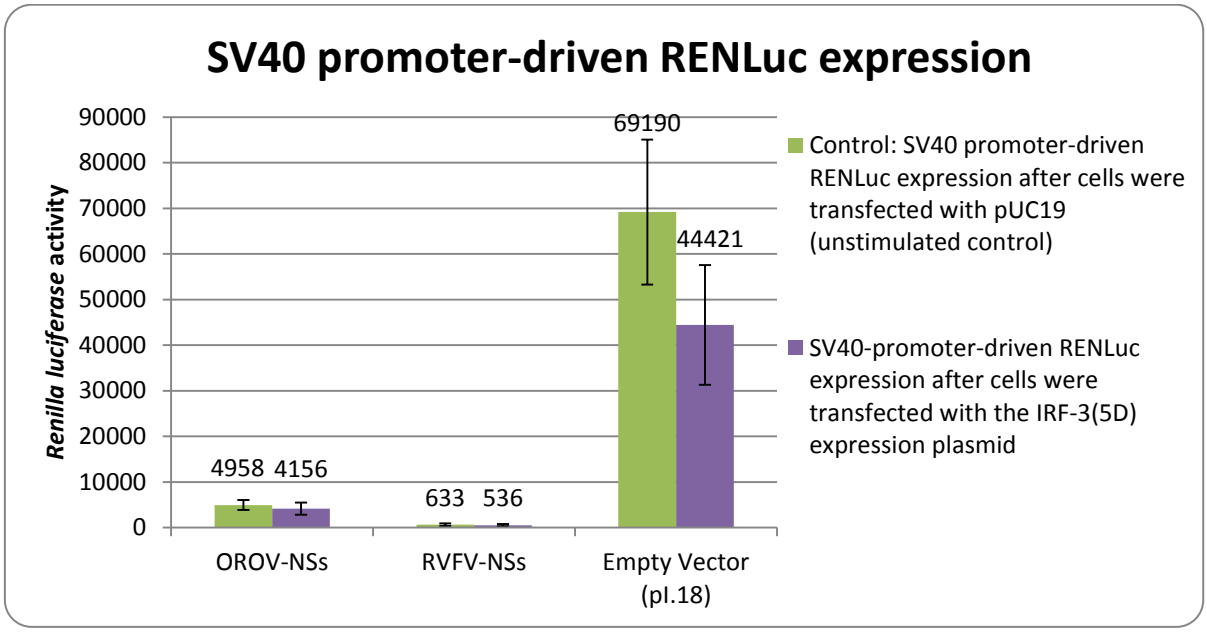

Figure 7. Renilla luciferase activity: internal control of transfection efficiency with pRL-SV40.

Green: Control (pUC19) **Purple: Stimulated (IRF-3(5D))**

Internal Control: Just as it was the case in the previous reporter assay the renilla gene expression was reduced (14 times lower) than that of the unstimulated control (Empty Vector) when the NSs protein of OROV (p = 0.0008) was present. The effect was even more pronounced (factor 109, p = 0.0006) when the RVFV-NSs protein was present.

3.1.1.3 **Transcription Factor NF-κB**

Objective:

Does the inhibitory effect of the OROV-NSs lie in inhibiting the activation of the transcription factor NF-κB? If the activation of NF-κB is artificially induced in a cell, do we see inhibition of the NF-κB-dependent reporter gene expression in the presence of the OROV-NSs protein?

Description:

16 hours after seeding 293 cells in a 12-well plate, 0.01 µg of TNF-α was used to stimulate the cells of some wells. Over a complex cellular cascade of reactions, the TNF-α stimulation results in the activation of the transcription factor NF-κB present in the cell and it also induces NF-κB gene expression (Nickles et al. 2012). The activated NF-κB binds to the artificial NF-κB-responsive promoter of the reporter plasmid p55A2-luc, inducing the expression of the firefly luciferase protein, whose activity is measured (see 2.2.10).

Experiment:

293 cells were transfected with the reporter plasmids p55A2-luc and pRL-SV40 (see 2.2.6). Three sets of experiments were carried out and are displayed beside each other on each of the charts (see **Figure 8** and **Figure 9**):

Set 1: The plasmid expressing the OROV-NSs protein (pI.18-OROV-NSs-FLAG) was transfected into the cells of two wells. 0.01 µg of TNF-α was added to the cells of one of these samples and nothing was added to the other sample, thus serving as the unstimulated control.

Set 2: The plasmid pI.18-OROV-NSs-FLAG in the above "Set 1" was replaced by the equivalent amount of the RVFV-NSs-expressing plasmid pI.18-RVFV-NSs-Z1. Everything else remained the same.

Set 3: The pI.18-OROV-NSs-FLAG in the initial experiment "Set 1" was replaced with the empty vector, pI.18, which served as the control.

All 6 wells with cells were transfected with p55A2-luc					
Cells were not stimulated (control)			Cells were stimulated with TNF-α		
OROV-NSs	RVFV-NSs	pI.18 (Empty Vector)	OROV-NSs	RVFV-NSs	pI.18 (Empty Vector)

Table 4: An overview of the plasmid combinations. To the cells of three wells TNF-α was not added (unstimulated) whereas TNF-α was added to the cells of the other three wells (TNF-α leads to the activation of NF-κB). The OROV-NSs-, the RVFV-NSs-expression plasmids and pI.18 (empty vector) were transfected into the cells of each of the three wells. This was carried out for the cells to which TNF-α was added and for the cells in the three wells to which no TNF-α was added. Firefly luciferase activity of the sample without TNF-α is represented by the blue bars, those with TNF-α by the red bars in Figure 8.

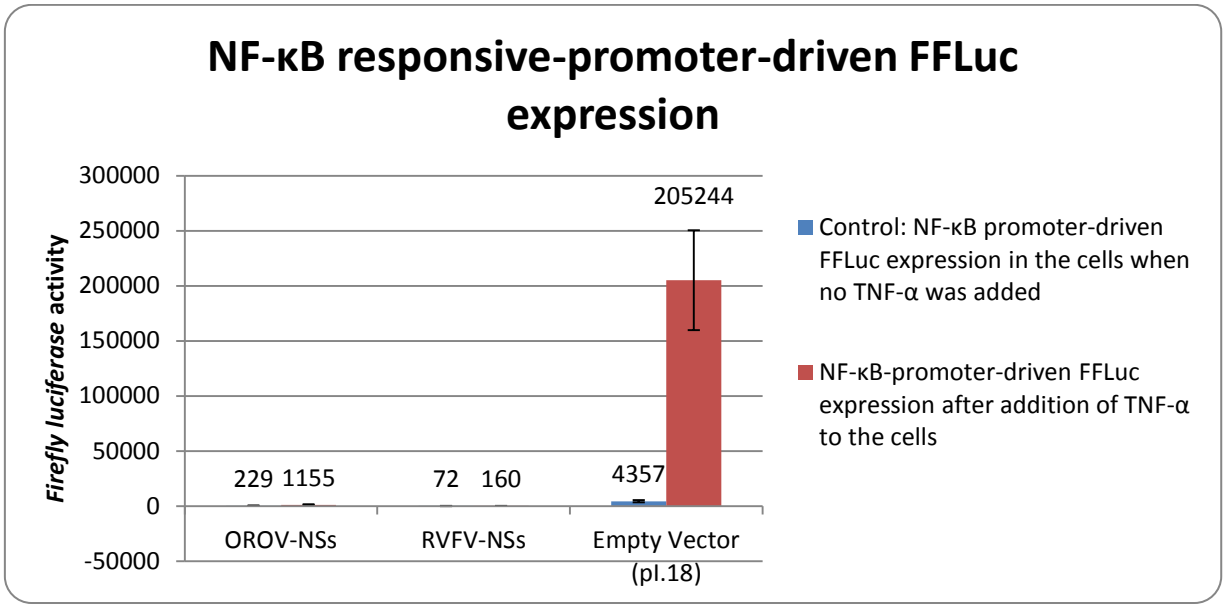

Figure 8. Firefly luciferase activity: effect of the OROV-NSs on NF-κB-dependent luciferase expression.

Blue: Control (Nothing added) **Red: TNF-α was added to these cells**

Empty Vector (Control): The cells that were stimulated with TNF-α showed an increase in its firefly luciferase activity by the factor 47 (p = 0.0005, see **Figure 8**, "Empty Vector"), indicating efficient activation of NF-κB by TNF-α.

RVFV-NSs and OROV-NSs: However, when this empty vector is exchanged for the expression plasmid expressing either the OROV- or RVFV-NSs protein, we do not see such a major, significant increment at all. Rather a decrease in control luciferase activity by factor 19 (see Figure 8, "OROV-NSs", p = 0.0006) and factor 60 (see Figure 8, "RVFV-NSs", two sided p = 0.0005) was observed in comparison to the control cells transfected with the empty expression vector. Firefly luciferase expression of the stimulated cells expressing OROV-NSs and the stimulated control (Empty Vector) differs by a factor of 177 (p = 0.0005).

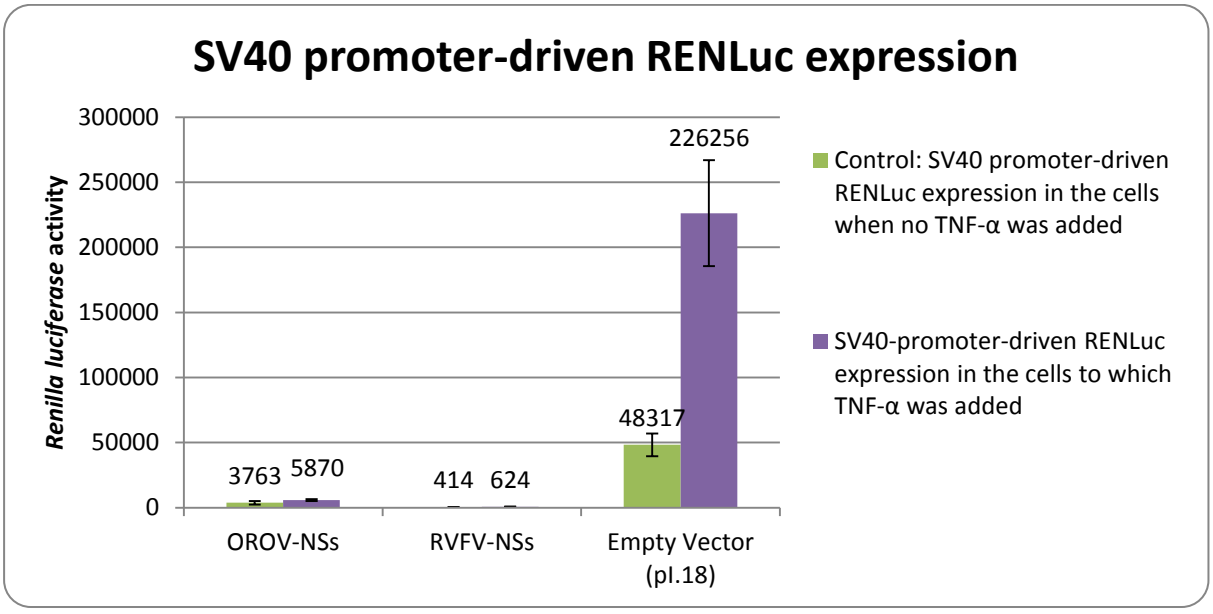

Figure 9. Renilla luciferase activity: internal control of transfection efficiency with pRL-SV40.

Green: Control (Nothing added) **Purple: TNF-α added to the cells**

Internal Control: Just as it was the case in the previous experiments, the renilla luciferase gene expression was inhibited by RVFV-NSs and OROV-NSs. When the NSs protein of OROV was present, the renilla luciferase expression level was only 0.08 ($p = 0.0003$) relative to the level of the unstimulated control (Empty Vector) and 0.008 relative to the level of the control when the RVFV-NSs protein was present ($p = 0.0002$).

3.1.1.4 Transcription Factor AP-1

Objective:

Does the inhibitory effect of the OROV-NSs lie in inhibiting the activation of the transcription factor ATF-2/cJun (AP-1)? In other words, if the activation of AP-1 is artificially induced in a cell (independent of its physiologic pathway) and the OROV-NSs is present, do we see inhibition of the AP-1-responsive reporter gene expression?

Description:

Here, a MEKK expression plasmid was transfected into the cells. MEKK expression is CMV-promoter-driven and is therefore constitutively active. It produces the kinase MEKK that kicks-starts an intracellular cascade that ends in the activation of the transcription factor AP-1 (Bernstein et al. 1994). The activated AP-1 binds to the artificial promoter of the co-

transfected reporter plasmid pAP1-luc, inducing the expression of the firefly luciferase protein, whose activity is measured (see 2.2.10).

Experiment:

293 cells were transfected with the reporter plasmid pAP1-luc and with the control plasmid pRL-SV40 (see 2.2.6). Three sets of experiments were carried out and are displayed beside each other on each of the charts (see **Figure 10** and **Figure 11**):

Set 1: The plasmid pI.18-OROV-NSs-FLAG was transfected into the cells of two wells. The MEKK-expression plasmid pFC-MEKK was transfected into the cells of one of these wells and an equivalent amount of pUC19 was transfected into the cells of the other well and served as the unstimulated control.

Set 2: The plasmid pI.18-OROV-NSs-FLAG in the above set was replaced by the plasmid pI.18-RVFV-NSs-Z1. Everything else remained the same.

Set 3: The pI.18-OROV-NSs-FLAG in the initial experiment (Set 1) was replaced by the empty vector, pI.18, which served as the control.

All 6 wells with cells were transfected with pAP1-luc					
Cells were transfected with pUC19 (unstimulated control)			Cells were transfected with pFC-MEKK (stimulation)		
OROV-NSs	RVFV-NSs	pI.18 (Empty Vector)	OROV-NSs	RVFV-NSs	pI.18 (Empty Vector)

Table 5: An overview of the plasmid combinations. The cells of three wells were transfected with pUC19 (unstimulated control) and the cells of the other three wells were transfected with pFC-MEKK (MEKK leads to AP-1 activation). The OROV-NSs-, the RVFV-NSs expression plasmids and pI.18 (empty vector) were transfected into the cells of each of the three wells. This was done for the cells of the three wells co-transfected with pFC-MEKK and the cells of the three wells co-transfected with pUC19 (control). The blue bars represent firefly luciferase activity of the control cells, that of the cells transfected with the pFC-MEKK by red bars in Figure 10.

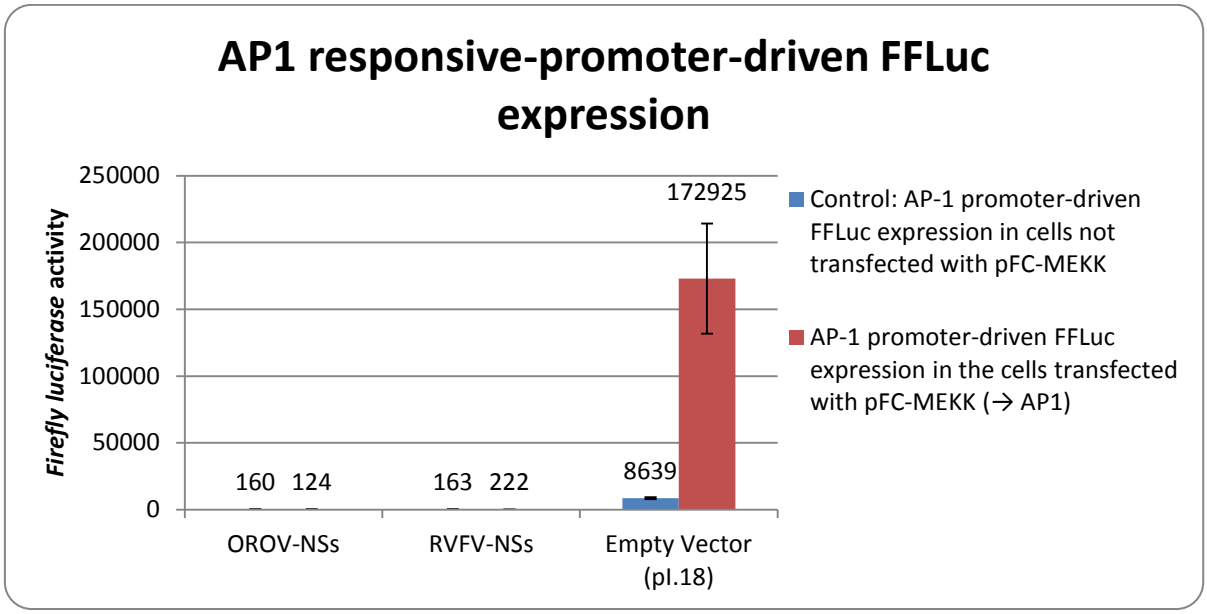

Figure 10. Firefly luciferase activity: effect of the OROV-NSs on the AP-1-dependent luciferase expression.

Blue: Control (pUC19) **Red: Cells transfected with MEKK expression plasmid (MEKK leads to AP-1 activation)**

Empty Vector (Control): The firefly luciferase activity increases by almost 20 times if the cells are co-transfected with pFC-MEKK (see **Figure 10**, "Empty Vector"). The activity difference is highly significant with a p value of 0.0008.

RVFV-NSs and OROV-NSs: When the empty vector was replaced by an expression plasmid for either the OROV-NSs or the RVFV-NSs protein, we did not see this increment at all, meaning that although we were artificially activating AP-1 in the cell, firefly luciferase gene expression was inhibited. The control firefly luciferase activity of the NSs-expressing cells also dropped, whereby reporter gene activity in the OROV-NSs- and RVFV-NSs-expressing cells was reduced by factor 53 ($p = 1.09 \times 10^{-5}$ and 1.12×10^{-5}). The cells that were transfected with both the OROV-NSs and MEKK expression plasmids showed a major (1395 factor) difference in reporter gene expression in comparison to the control cells transfected with pI.18 and the MEKK expression plasmid (with a p value of 0.0007).

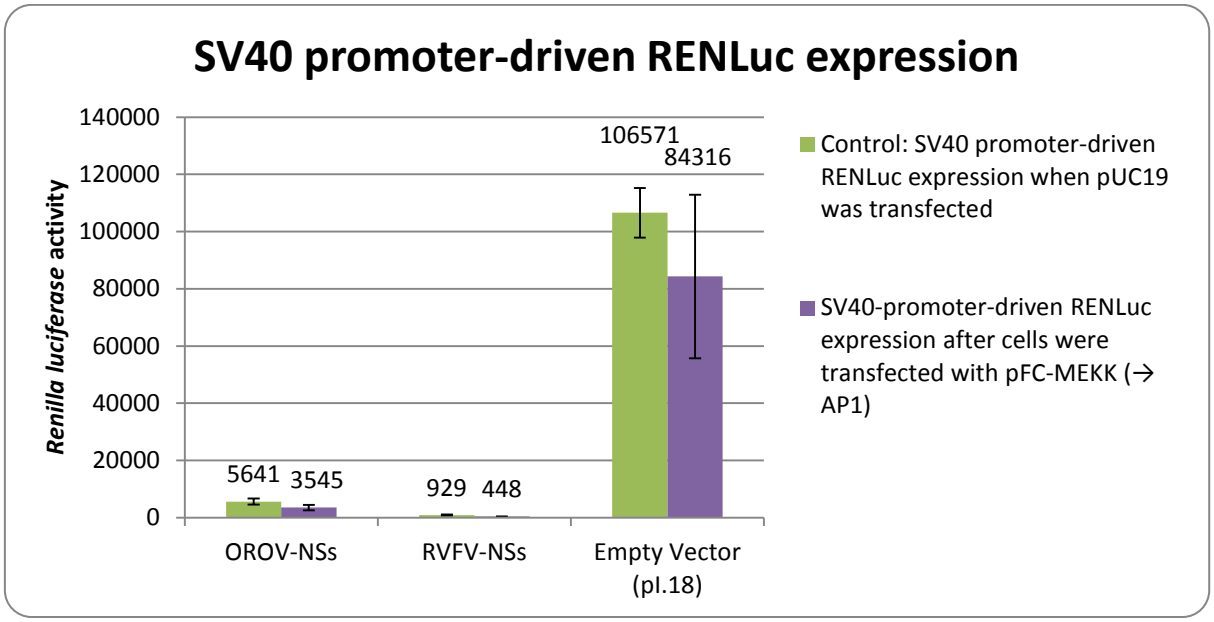

Figure 11. Renilla luciferase activity: internal control of transfection efficiency with pRL-SV40.

Green: Control (pUC19) Purple: Cells transfected with the MEKK expression plasmid (MEKK leads to AP-1 activation)

Internal Control: Just as it was the case in all of the above experiments, the renilla luciferase gene expression was efficiently inhibited in the presence of OROV-NSs and RVFV-NSs. It fell to 0.05 of the reporter gene expression seen in the control cells transfected with pUC19 (see **Figure 11**) when the NSs protein of OROV was present (with p = 0.00001) and even to 0.009 of the control when the RVFV-NSs protein was present (with p = 0.00001).

3.1.1.5 Inhibitory Effects of OROV-NSs on Transcription and Translation

Cap-*independent* Translation:

Objective:

Does the inhibitory effect of the OROV-NSs protein on the reporter gene expression (IFN-β-, IRF-3-, NF-κB-, AP-1-responsive and SV40 promoter-driven expression) occur on the level of transcription or translation?

Description:

The level of transcription can be skipped in a cell if a reporter plasmid is *in vitro* transcribed into RNA and this RNA then transfected into a cell instead of the DNA form of the plasmid. This *in vitro* (cell-free) RNA generation (see 2.2.7) replaces the cellular RNA polymerase II-

dependent transcription. Transfection of the *in vitro* transcribed RNA (see 2.2.9) makes the expression of the luciferase gene, which is encoded on the RNA, independent of RNAP II and solely dependent on translational processes. Ribosomes interact with the 5' cap end of mRNA: the 40S ribosomal subunit binds to this cap region and from this point onwards, RNA translation occurs. Alternatively, ribosomes can bind to an Internal Ribosome Entry Site (IRES), which are RNA regions that can directly recruit the 40S ribosomal subunit in a cap-independent way, resulting in RNA translation (López-Lastra et al. 2005).

The DNA plasmid pTM1-FFLuc was chosen because it contains an expression cassette consisting of a T7 RNA polymerase promoter followed by an IRES and the FFLuc cDNA. It was transcribed *in vitro* to RNA and transfected into BSR-T7 cells. Standard guanosin was used for the *in vitro* transcription, i. e. the transcripts are not capped at the 5' end.

Experiment:

0.5 µg of this pTM1-FFLuc RNA was transfected into the BSR-T7 cells of each of four wells. Into the cells of each of these wells, an increasing amount of the OROV-NSs expression plasmid was transfected, starting by transfecting no OROV expression plasmid into the cells of the first well (control), followed by 100 ng, 250 ng, and ending with 500 ng of plasmid. To make sure that the total amount of nucleic acid always remained constant between these four wells, the RVFV Clone 13 NSs expression plasmid was transfected to compensate for the missing nucleic acid when the amount of transfected OROV-NSs expression plasmid was varied. In the end,

1 µg of nucleic acid was always transfected. The RVFV Clone 13 NSs expression plasmid (see 2.1.8) expresses the mutated RVFV Clone 13 NSs, which has no inhibitory function on RNA polymerase II-mediated expression (Vialat et al. 2000).

All cells were co-transfected with the control plasmid pRL-SV40 DNA. The experiments were carried out three times to attain significant results.

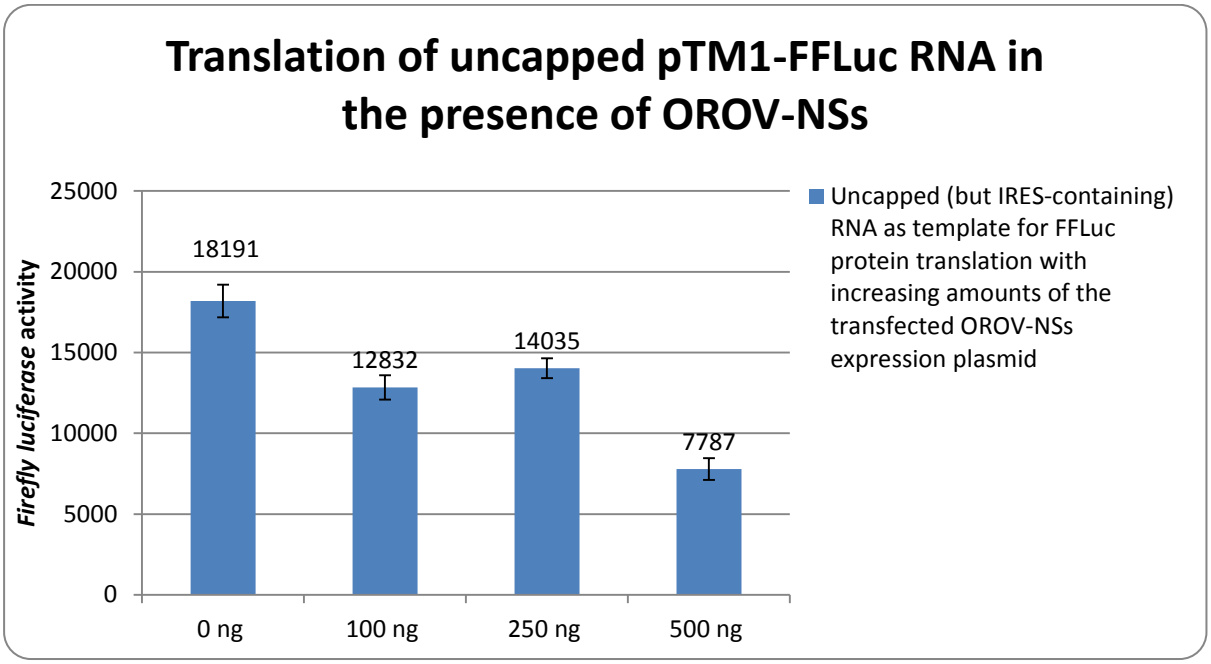

Figure 12. Firefly luciferase activity: increasing amounts of the OROV-NSs expression plasmid was co-transfected together with a constant amount of the luciferase expressing RNA, which was transcribed *in vitro* using pTM1-FFLuc as template. X-axis = respective OROV-NSs expression plasmid amounts transfected into the cells.

The firefly luciferase activity dropped by only 30 % (compared to its control activity) when 100 ng of the OROV-NSs expression plasmid was transfected ($p = 0.001$, see Figure 12). In contrast, the renilla luciferase activity ($p = 0.0004$, see **Figure 13**) dropped 24 times when 100 ng of the OROV-NSs expression plasmid was co-transfected in comparison to its control activity in the absence of OROV-NSs. When 500 ng of the OROV-NSs expression plasmid was transfected into the cells the firefly luciferase activity was only 2.3 times lower ($p = 0.0006$) compared to the control without OROV-NSs (see Figure 12, “500 ng” versus “0 ng”) whereas its renilla luciferase counterpart was 46 times lower ($p = 0.0004$). Although the 2.3 factor drop in firefly luciferase activity is significant, it was far from the 46-fold reduction seen in the RNA-polymerase II-dependent renilla luciferase reporter gene expression (see Figure 13).

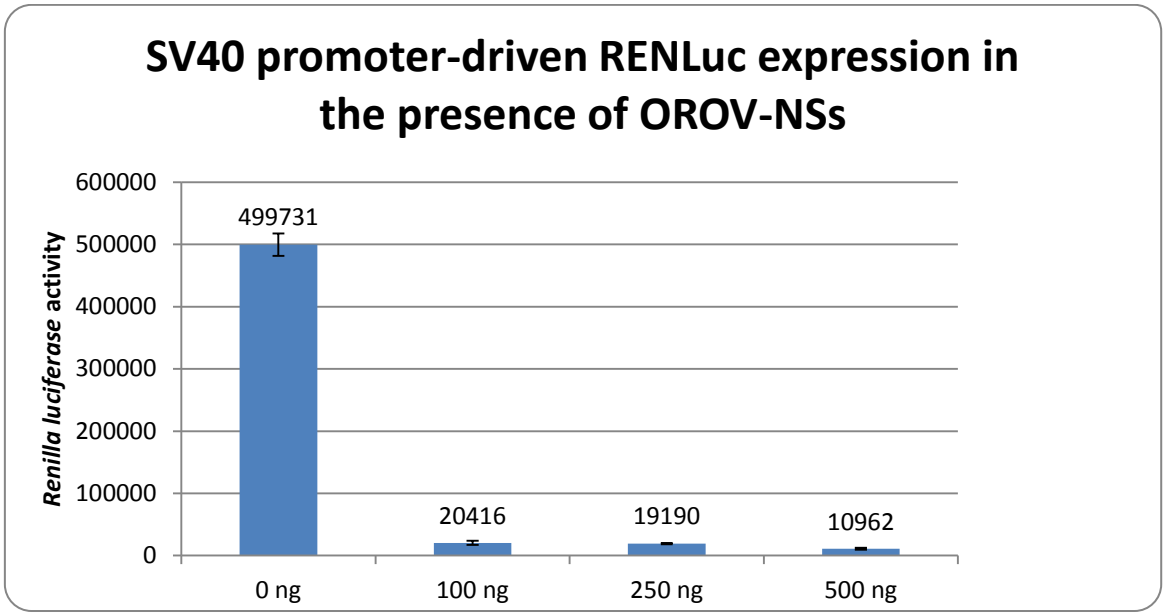

Figure 13. Renilla luciferase activity: with increasing amounts of the transfected OROV-NSs expression plasmid, the renilla luciferase gene expression (pRL-SV40) is increasingly inhibited. X-axis = respective OROV-NSs expression plasmid amounts.

Cap-*dependent* Translation:

Objective:

Could it be that the OROV-NSs does indeed inhibit translation by specifically interfering with the cellular cap-dependent method of translation?

Description:

Eukaryotic cells cap their RNA in the cell nucleus before extranuclear transportation and the translation machinery recognizes this cap and begins protein synthesis. It could be that the OROV-NSs protein does indeed inhibit translation, in contrast to the findings of the previous experiment (see 3.1.1.5., “Cap-independent Translation”), and not transcription, by inhibiting specifically the translation of capped RNA (and not IRES-dependent translation). It was therefore necessary to test if the expression of an artificially generated capped reporter RNA was inhibited in the presence of the OROV-NSs.

Experiment:

The DNA plasmid pT7-riboSM2-cMpro cRL served as the template for *in vitro* transcription. This plasmid that lacks an IRES was transcribed *in vitro* to RNA (see 2.2.6), this time however, a part of the ribonucleotide GTP was replaced with its capped form (7mG(5’)ppp(5’)G): instead

of the 2.5 mM GTP normally added, a mixture of 2 mM of its cap analogue and 0.5 mM of standard GTP was added. The cap analogue to normal GTP ratio was 4:1.

The *in vitro* transcribed cMpro cRL RNA was transfected into BSR-T7 cells. An increasing amount of the OROV-NSs expression plasmid was transfected into the cells of the respective wells, starting from transfecting none (control), to 100 ng, 200 ng, and 400 ng in the last well. The total amount of nucleic acid remained constant between the cells of the different samples by transfecting the RVFV-NSs Clone 13 expression plasmid, such that at the end 1 µg of nucleic acid (0.5 µg DNA + 0.5 µg RNA) was always transfected. The cells were always co-transfected with 100 ng of the constitutively active pGL3-FFLuc DNA control plasmid.

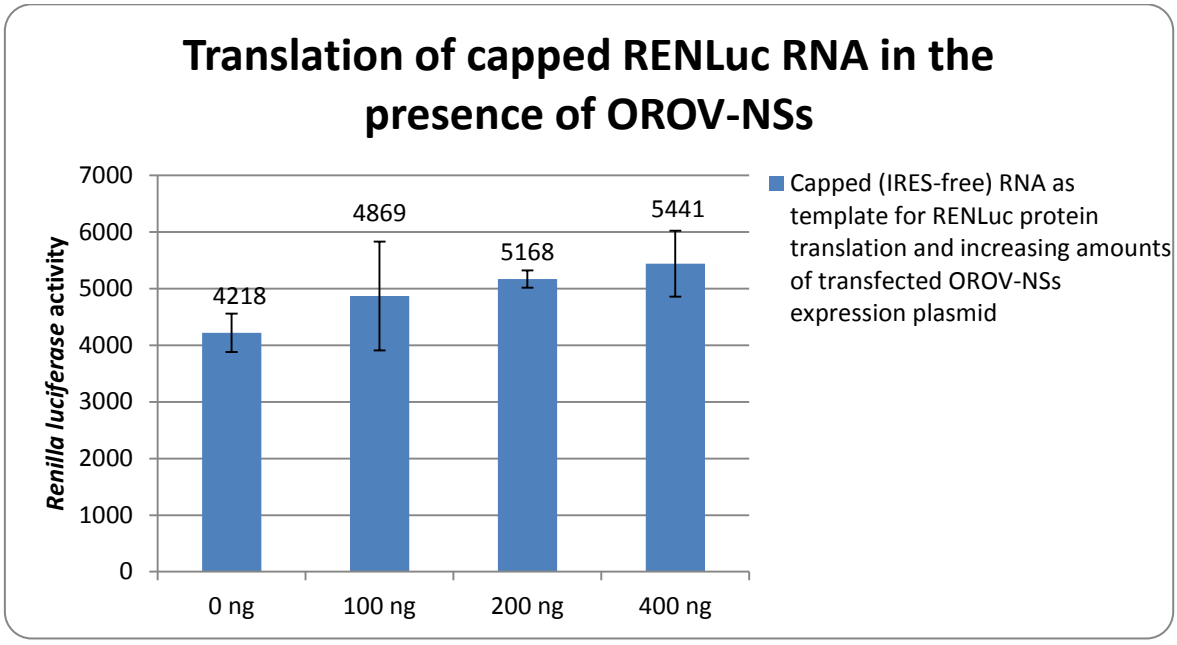

Figure 14. Renilla luciferase activity: increasing amounts of the OROV-NSs expression plasmid were co-transfected with a constant amount of the renilla luciferase expressing RNA. X-axis = respective OROV-NSs expression plasmid amounts transfected.

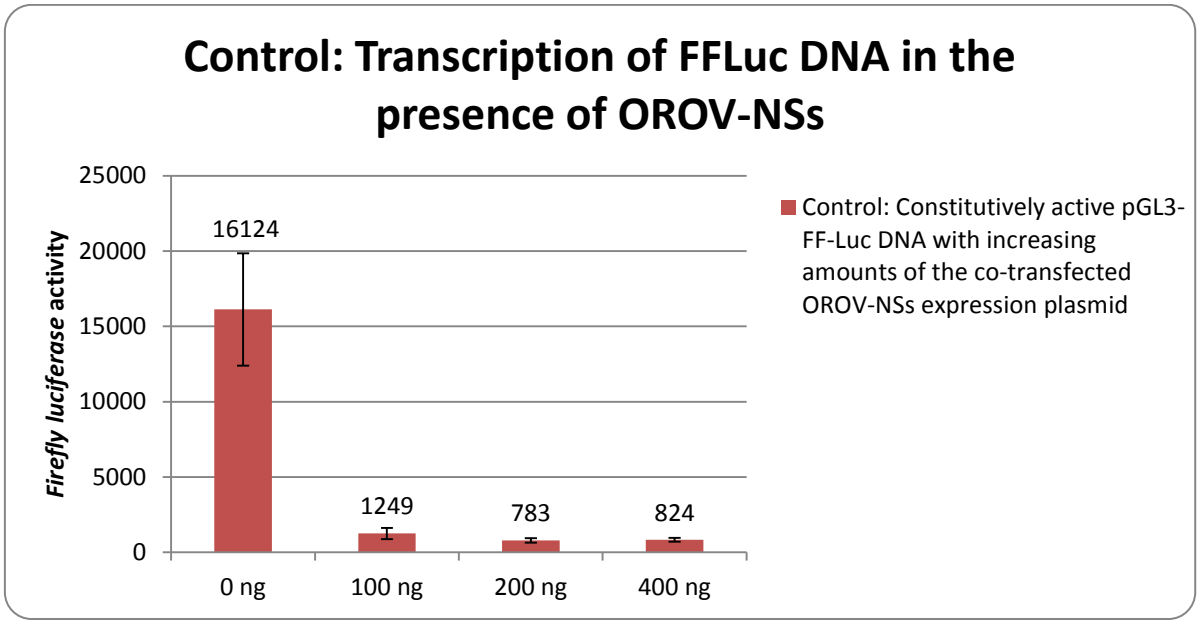

Figure 15. Firefly luciferase activity: with increasing amounts of the transfected OROV-NSs expression plasmid, the firefly luciferase gene expression is increasingly inhibited. X-axis = OROV-NSs expression plasmid amount transfected.

The cells that were transfected with 100 ng of the OROV-NSs expression plasmid showed a significant reduction (factor 13, p = 0.02) in the firefly luciferase activity when compared to the cells into which no OROV-NSs expression plasmid was transfected (see Figure 15). There was no inhibition at all observed for the translation of capped RNA in the cells expressing OROV-NSs (see Figure 14). There was even a slight, but significant increase in the renilla luciferase expression to be seen when the cells were transfected with 400 ng of the OROV-NSs expression plasmid and the renilla luciferase expression was compared to that of the cells not expressing the OROV-NSs (factor 1.3, p = 0.05).

3.1.1.6 **Effects of the OROV-NSs on the T7 DNA dependent RNA-polymerase**

Objective:

Does the OROV-NSs protein inhibit the activity of the T7 polymerase that is expressed in BSR-T7 cells?

Description:

BSR-T7 cells constitutively express the T7 polymerase and this polymerase transcribes expression cassettes that have the T7 promoter. The expression cassette of the plasmid pTM1-FFLuc has such a promoter.

Experiment:

0.5 µg of the pTM1-FFLuc DNA plasmid was transfected into the BSR-T7 cells. Into each of the wells containing these cells, an increasing amount of the OROV-NSs expression plasmid was transfected, starting from transfecting none (control), to 100 ng, 250 ng, and ending by transfecting 500 ng in the cells of the last well. The total amount of transfected nucleic acid remained constant between the cells in the different wells by transfecting the RVFV-NSs Clone 13 expression plasmid, such that at the end, 500 ng of nucleic acid was always transfected.

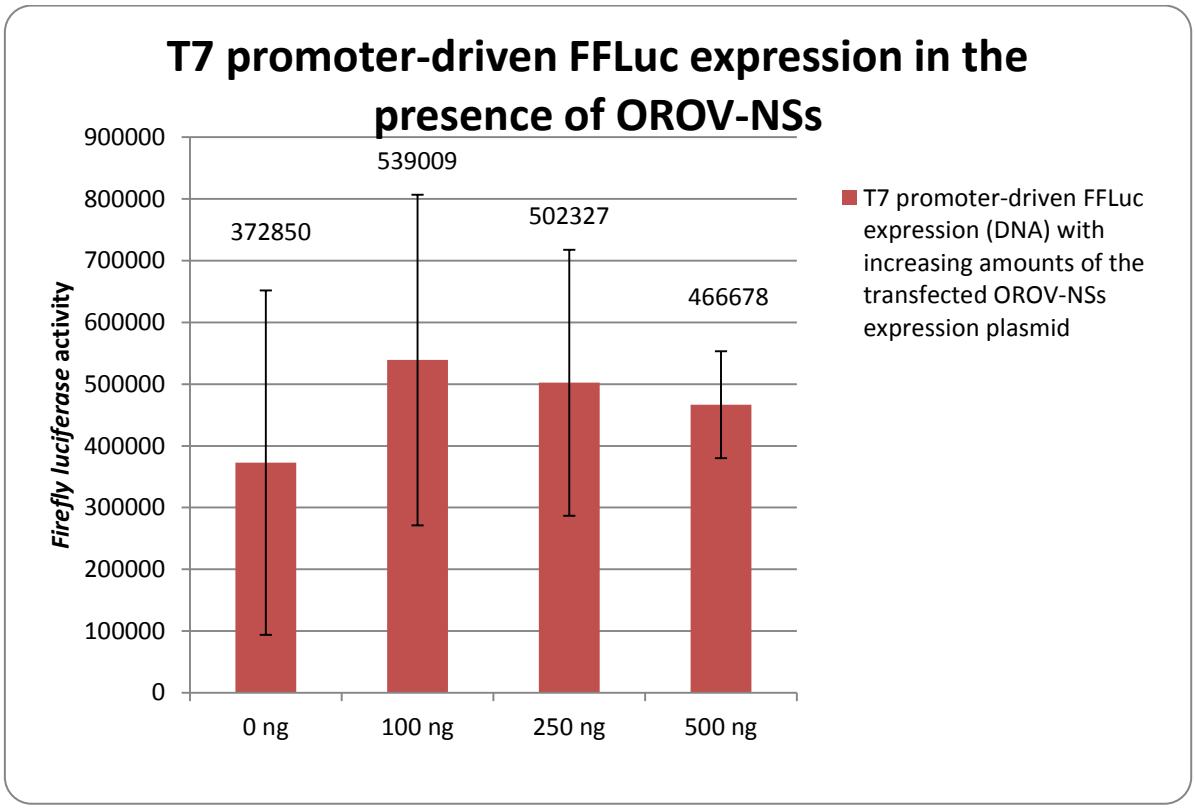

Figure 16. Firefly luciferase activity: increasing amounts of the OROV-NSs expression plasmid were co-transfected together with a constant amount of the luciferase expression DNA plasmid pTM1-FFLuc. X-axis = OROV-NSs expression plasmid amounts transfected.

There is no significant inhibition of the expression of the pTM1-FFLuc DNA reporter plasmid to be seen when increasing amounts of the OROV-NSs expression plasmid was transfected into

the cells (see **Figure 16**). This is in contrast to the effects the OROV-NSs has on the SV40 promoter-driven reporter gene expression (plasmid pRL-SV40) in the preceding experiments which is dependent on the eukaryotic RNA-polymerase II.

3.1.2 Effects of OROV-NSs on Phosphorylation State of RNAP II

Objective:

Does the OROV-NSs protein reduce transcription activity by preventing the phosphorylation of the cellular RNA polymerase II? Alternatively, could it be that the NSs induces degradation of the polymerase?

Description:

The cellular DNA-dependent RNA polymerase II (RNAP II) is the synthesizer of mRNA. The C-terminal domain (CTD) of the polymerase consists of 52 repeats of the sequence YSPTSPS. For the initiation of transcription of a gene, the RNAP II has to be phosphorylated at serine 5 of these repeats. Once the TFIIH kinase has catalyzed multiple of the serine 5 phosphorylations of the many repeats, the polymerase initiates transcription.

The serine at position 2 of these repeats additionally has to be phosphorylated after initiation for the polymerase to carry out the elongation and the 3'-end processing of the transcript (Thomas et al. 2004).

Antibodies are used in the following immunofluorescence experiments that bind specifically to either phospho-serine 5 (initiation) or to phospho-serine 2 (elongation/3'-end processing). The antibody against phospho-serine 5 is called H14 and that against phospho-serine 2, H5. Secondary antibodies that are coupled to fluorescent dyes bind to the first antibodies. The coupled fluorophore emits fluorescent signals of a particular wavelength after light stimulation (see 2.2.11).

A third primary antibody was used: the N20 antibody is an antibody that recognizes the N terminus of the large subunit of the RNAP II irrespective of the CTD phosphorylation state. It targets the active and inactive polymerase alike.

If for example, the serine at position 5 of the repeats is not phosphorylated, the H14 antibody has nothing to bind to and is washed away, leaving a dark cell nucleus when the cells are stimulated with light under the confocal microscope.

The following experiments involved transfecting Vero E6 cells with the NSs expression plasmids of either the OROV, the RVFV or with a control plasmid that is used for the expression of a protein that has no influence on CTD phosphorylation (FLAG-ΔMx). Each of these proteins carries a FLAG tag, against which antibodies have been generated. This so-called anti-FLAG antibody was the fourth primary antibody used.

Experiment:

(See 2.2.11) The secondary bodies used in all of the following experiments were the CY3-conjugated anti-mouse antibody and the FITC-conjugated anti-rabbit antibody. The DNA dye To Pro 3 Iodide was always added to the primary and secondary antibody dilutions. To Pro 3 stains DNA in the cell nucleus. The group of cells transfected with the RVFV-NSs expression plasmid served as the reference, since a lot is known about the RVFV-NSs inhibition mechanism.

Effects of NSs on transcription initiation (Figure 17):

Vero E6 cells were transfected with the OROV-NSs, the RVFV-NSs expression plasmid or the FLAG-ΔMx plasmid. 24 h later, both of the following primary antibodies were added: the mouse antibody H14 (that binds to phospho-serine 5) together with the rabbit anti-FLAG polyclonal antibody (that binds to the NSs-FLAG). After a 1 h incubation period, the cells were washed and subsequently incubated with the secondary antibodies for another hour.

Effects of NSs on transcript elongation (Figure 18):

Vero E6 cells were transfected with the OROV-NSs, the RVFV-NSs expression plasmid or with the FLAG-ΔMx plasmid. 24 h later, both of the following primary antibodies were added: this time, the mouse antibody H5 (that binds to phospho-serine 2) was used instead of the H14 antibody, together with the rabbit anti-FLAG polyclonal antibody that binds to NSs (FLAG region). After a 1 h incubation period, the cells were washed and subsequently incubated with the secondary antibodies for another hour.

Effects of NSs on the total RNAP II amount (Figure 19):

24 h after transfection with the OROV-NSs, the RVFV-NSs expression plasmid or the FLAG-ΔMx plasmid, the following primary antibodies were added: the rabbit N20 antibody that binds to active or inactive RNAP II alike and the mouse monoclonal anti-FLAG M2 antibody that binds to NSs (FLAG region). After a 1 h incubation period, the cells were washed and subsequently incubated with the secondary antibodies for another hour.

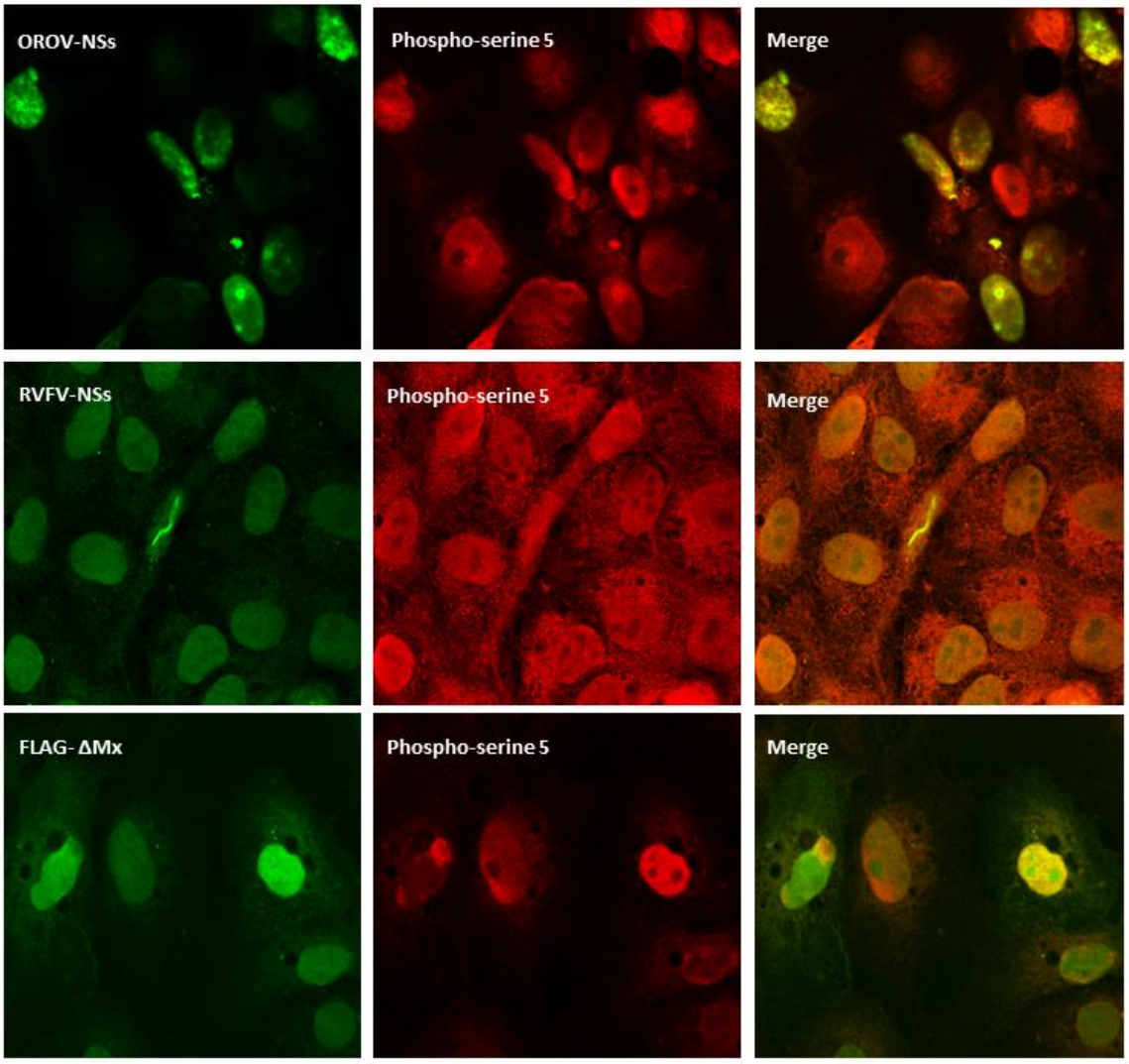

Figure 17. Green represents the NSs protein of OROV, RVFV or the protein FLAG-ΔMx. Red represents the phosphorylated serine 5 of the CTD of the RNAP II (initiation of transcription).

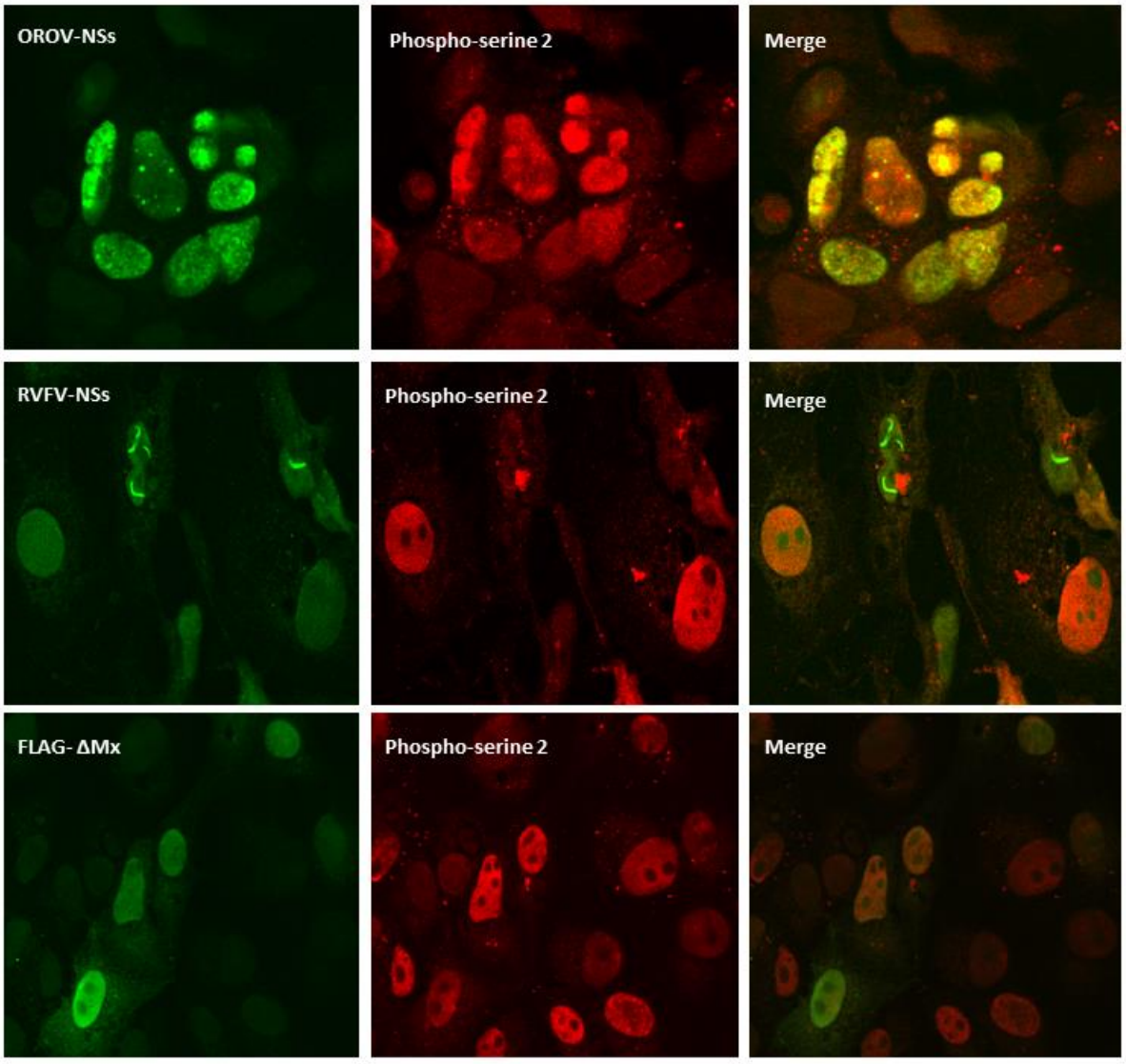

Figure 18. Green represents the NSs protein of OROV, RVFV or the protein FLAG-ΔMx. Red represents the phosphorylated serine 2 of the CTD of the RNAP II (elongation).

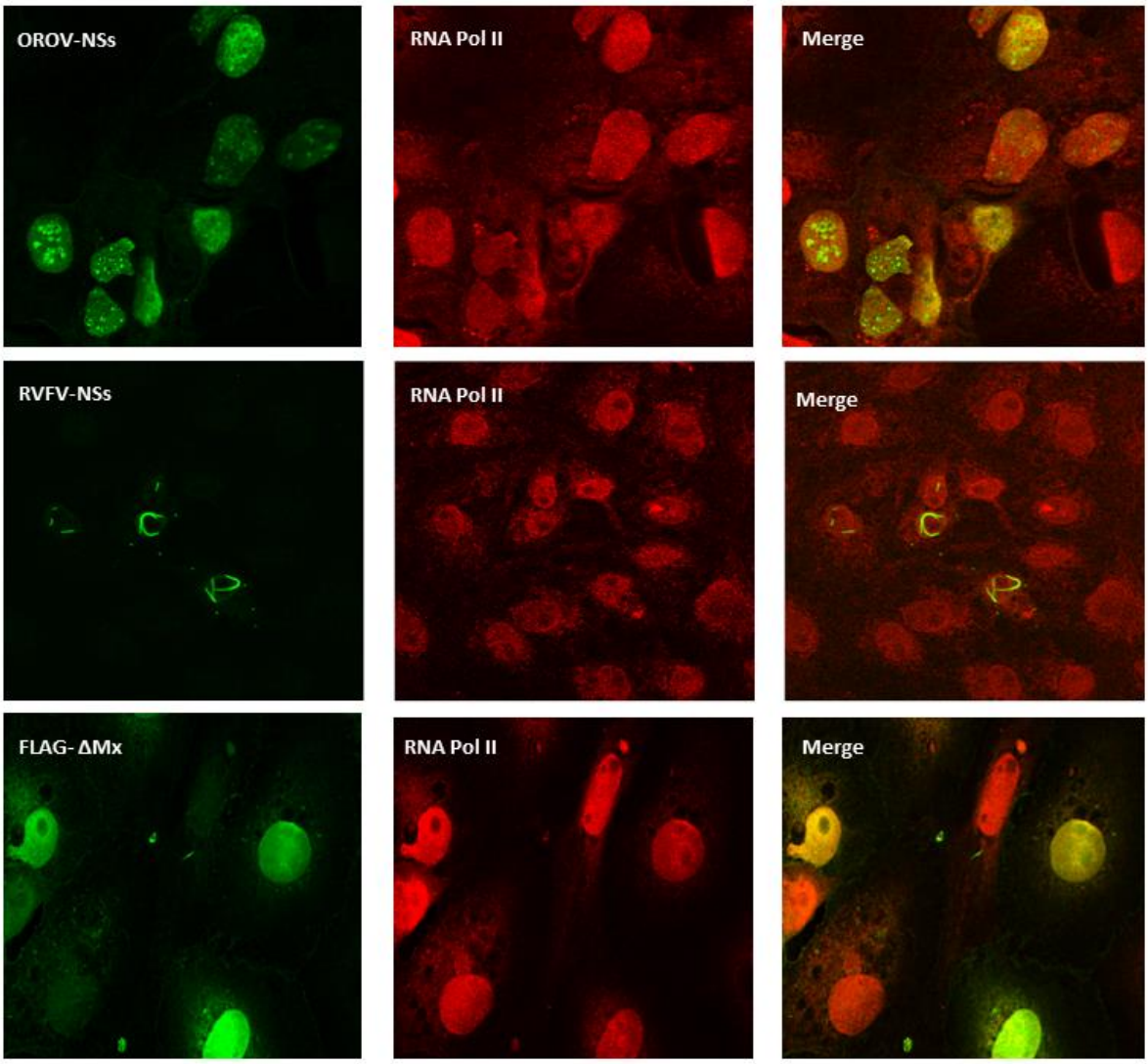

Figure 19. Green represents the NSs protein of either OROV, RVFV or the protein, FLAG-ΔMx. Red represents the RNAP II.

In all images, green represents a protein that has a FLAG region, so the NSs proteins (either that of the OROV or RVFV) or the control (FLAG-containing non-functional protein). Red is either the phosphorylated (H5 and H14 antibodies) or the inactive/active RNAP II (N20 antibody). The "Merge" images are the fusion of both images to show co-localization. Yellow color on these merged images therefore represents an overlay of the green NSs (FLAG) with the red RNAP II, which means both cellular bodies to which the antibodies have bound, are in the same place.

When closely examined, the OROV-NSs proteins seem to create structures circular in shape in the cell nucleus (see Figures 18, 19).

No reduction in the phosphorylation state of serine 2 or serine 5 in the RNAP II is visible in the cells expressing the OROV-NSs protein (see Figures 17 and 18). There is also no weakening of the N20 signal in these cells. This means there is neither inactivation nor apparent degradation of the RNAP II in OROV-NSs-containing cells (see Figure 19).

The images seem to show a geometrical association between the OROV-NSs and the active RNAP II. High signal intensity OROV-NSs nuclear regions on Figure 17 and 18 (upper panel of pictures, respectively) are overlaid by high intensity signals of active (= phosphorylated) RNAP II. In Figure 19, no obvious association is seen between the total RNAP II (both active and inactive) and the OROV-NSs.

Figures 17 to 19 also show the typical filamentous structure of intranuclear RVFV-NSs, which has previously been described (Billecocq et al. 2004).

In the cells expressing the RVFV-NSs protein there was apparently no inhibition of transcription initiation by the RNAP II (phospho-serine 5). The primary antibodies against phospho-serine 5 always found their target in the presence of RVFV-NSs (see Figure 17). Rather, elongation and/or 3'-end processing of the transcript is prevented by the RVFV-NSs: the H5 antibodies against phospho-serine 2 in Figure 18 were unable to bind to their targets in the cells transfected with the RVFV-NSs expression plasmid. The cells on the same image without the filamentous RVFV-NSs had enough phospho-serine 2 for high intensity signals.

The control images (see Figure 19) all show an even distribution of pI.18-FLAG-ΔMx in the cell nuclei irrespective of what primary antibody was used. This distribution of protein across the cell is what is normally expected. No ring-like (OROV-NSs) or filamentous (RVFV-NSs) structures were seen in any of the control cells.

3.2 The OROV Minireplicon System

Objective:

Previously, the Virology Institute of the Georg-August University in Göttingen set up a minireplicon system for the OROV. This has never been done before for the OROV. This system

was however inactive. Aim of the following experiments was to find the problem that prevented the OROV minireplicon system from working (see 2.2.12).

Experiment:

Reporter assays were carried out as described (see 2.2.12), whereby two sets of experiments were set up. The OROV minireplicon system was set up in both sets of experiments parallel to that of the La Crosse virus (LACV). The LACV minireplicon system is properly established; it is known to work and served as the control (Blakqori et al. 2003).

OROV minireplicon (Figure 20/C and 22/C): BSR-T7 cells were transfected with pTM1-orov-cSmut that is used for the expression of the nucleoprotein of the OROV (= OROV-N) and pTM1-orov-cL that is used for the expression the L protein (RNA polymerase; OROV-L) of the OROV. Both of these genes are T7 promoter-driven and are therefore constitutively expressed in T7 polymerase-expressing BSR-T7 cells. The plasmid pT7-riboSM2-orov-vMpro-vRL, which is used for the expression of the OROV minigenome, was co-transfected into the same cells. Recognition of the minigenome by the OROV-L and –N proteins lead to the expression of the renilla luciferase protein, whose gene is encoded on the minigenome.

The renilla luciferase activity of the OROV minireplicon was measured and compared to that of the LACV.

LACV minireplicon (Figure 20/A and 22/A): The LACV minireplicon involved transfecting BSR-T7 cells with pTM-LACV-L (used for the expression of the RNA polymerase of the LACV), pTM-LACV-N (used for the expression of the LACV nucleoprotein) and the plasmid pT7-ribo-LACV-vMpro-vRL, which is used for the expression of the LACV minigenome. The activity of the LACV minireplicon correlates with the expression of the renilla luciferase protein, whose activity is measured (see 2.2.10).

The controls for both minireplicon systems were carried out by replacing the transfected plasmids with an equivalent amount of pUC19 DNA (see Figure 20/22 for replaced plasmid). Furthermore, to show proper handling and efficient transfection, all cells were always co-transfected with the control plasmid pTM1-FFLuc (= used for the expression of the firefly luciferase). The firefly luciferase gene is also T7 promoter-controlled and is therefore constitutively expressed in BSR-T7 cells.

In the first set of experiments (Figure 20 and 21; see also 2.2.12 and Table 2) 0.3 µg of each plasmid was transfected per well. The renilla luciferase activity of the LACV minireplicon in comparison to the control was very high showing an active system. The OROV minireplicon on the other hand could not show significantly higher renilla luciferase activity in comparison to its control (see Figure 20/C) showing an inactive system. Its activity was far from that of the LACV minireplicon.

The LACV-L and –N expression plasmids were co-transfected together with the OROV minigenome expression plasmid (see 20/B1). This co-transfection revealed some interesting results (see Figure 20/B). Apparently, the RNA polymerase and nucleoprotein of the LACV, to some extent, recognized the OROV minigenome and this system lead to a greater expression of the renilla luciferase protein in comparison to the OROV system with only OROV components. The activity was however still a lot lower than that of the LACV minireplicon.

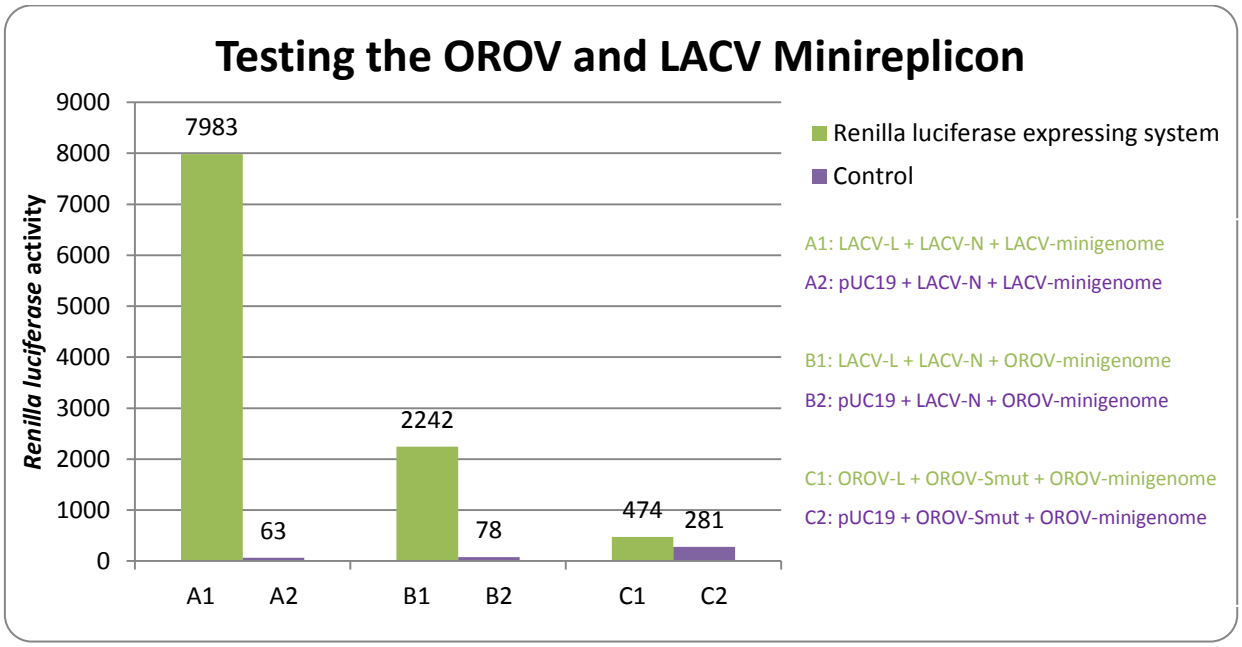

Figure 20. Renilla luciferase activity.

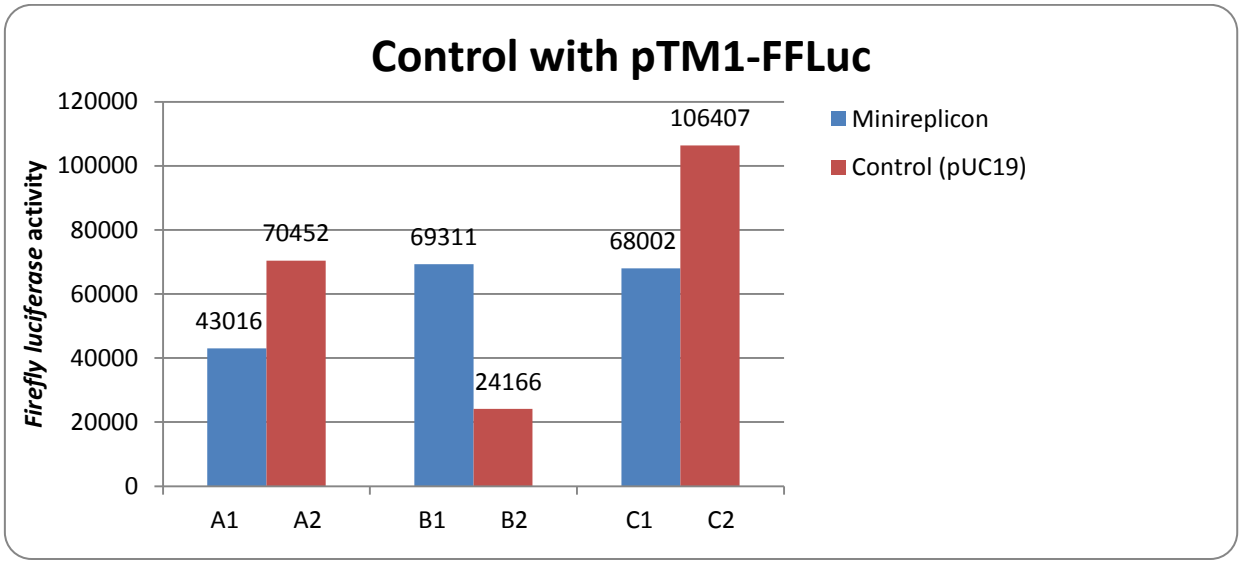

Figure 21. Firefly luciferase activity: internal control of transfection efficiency with pTM1-FFluc. A, B, and C correspond to A, B, and C in Figure 20, respectively.

A second set of experiments (Figure 22 and 23; see also 2.2.12 and Table 3) was carried out with further variation of the components and an increase in the amounts of transfected plasmid from 0.3 µg to 0.6 µg per well. The LACV-N expression plasmid in the LACV minireplicon was replaced by the OROV-N expression plasmid. This resulted in a major drop in minireplicon activity (see Figure 22/B) indicating that the nucleoprotein of the OROV was unable to recognize the LACV components. Like in the above set of experiments, the OROV minireplicon was barely active (see Figure 22/C).

The LACV nucleoprotein and the OROV RNA polymerase expression plasmids, and the OROV minigenome were then co-transfected together. Here too, the minireplicon was inactive (see Figure 22/D).

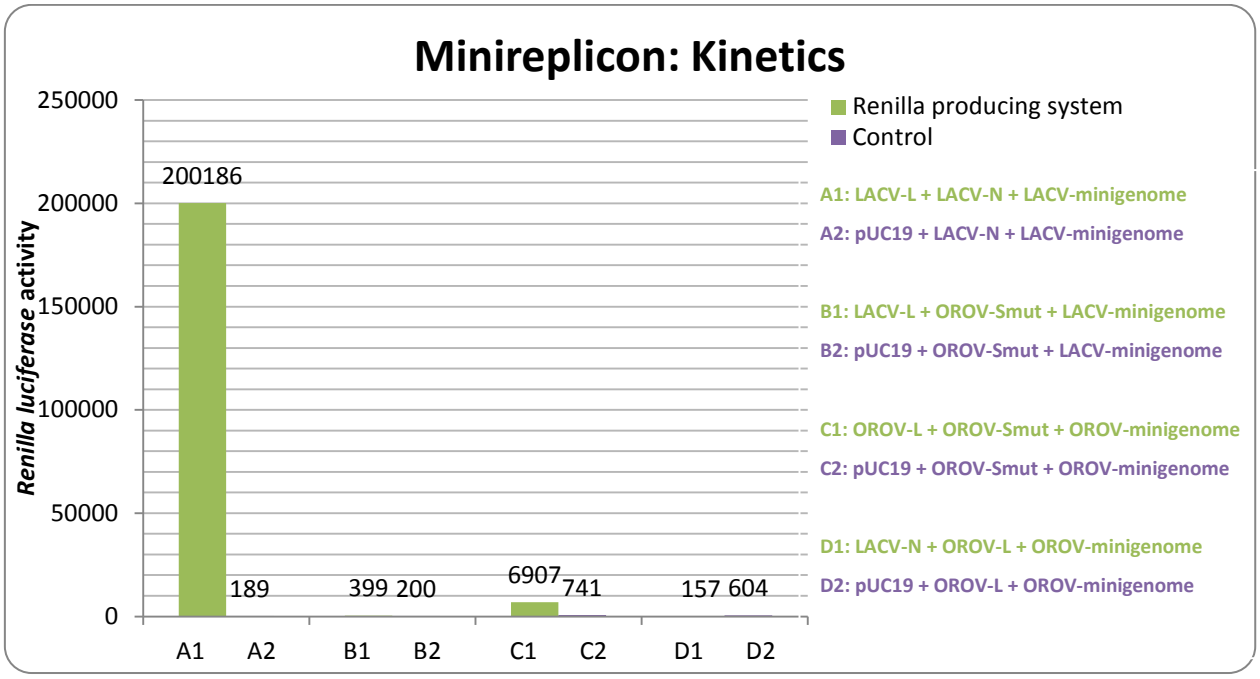

Figure 22. Renilla luciferase activity. A to D show the different systems set up.

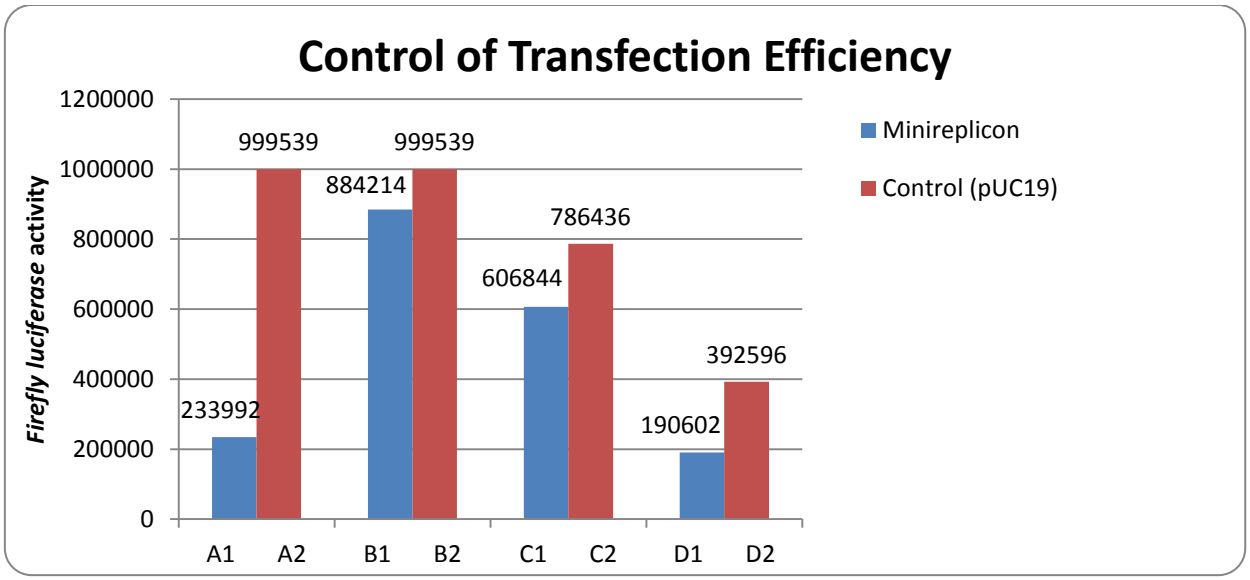

Figure 23. Firefly luciferase activity: internal control of transfection efficiency with pTM1-FFLuc. A, B, C and D correspond to A, B, C and D in Figure 22, respectively.

The control transfection with pTM1-FFLuc showed efficient transfection (see Figures 21 and 23) in all sets of experiments. Notable, is the increase in firefly luciferase expression in the pUC19 control sets.

Sequencing the OROV Minireplicon Components

In further efforts to identify the problem, pTM1-orov-cL was sequenced (Seqlab Göttingen GmbH). The sequence was compared to that of the OROV (the Trinidad strain) that is in the international gene bank. It turned out that in the OROV-L gene, there were indeed six nucleotide insertions in comparison to the OROV sequence in the gene bank. The first five inserted nucleotides were adenosines and in approximately the same region of the L segment at positions 2936, 2945, 2949, 2968 and 2974. A very close-by thymine was also inserted at position 2980. These changes mean the addition of two amino acids in the protein sequence.

Furthermore, the ORF of the OROV minigenome (i. e. the RENLuc cDNA) was sequenced and found to be correct. The 3`and 5`NTRs of the minigenome were initially not sequenced because they require special procedures due to the complement bonds they build that gives the minigenome its pan-like structure. After the conclusion of experiments for this thesis, it was found that both NTR sequences were correct in comparison to the international gene bank. There was however one additional nucleotide when the sequence was compared to those of the other Orthobunyaviruses. This was very unusual because this is a highly conserved region amongst the Orthobunyaviruses (Keisers).

4 Discussion

4.1 Interference of OROV-NSs with IFN-β Promoter Activation

4.1.1 Effects of OROV-NSs on IFN-β Promoter Activation

The activation of the IFN-β promoter and subsequent IFN-β gene expression is a critical reaction of cells in response to viral invasion.

Previous experiments have shown that OROV, similar to other Orthobunyaviruses, inhibits the expression of IFN-β in infected cells and that the viral NSs protein is responsible for this inhibition (Keisers). Overexpression of IRF-3(5D) in a cell can activate the IFN-β promoter (Hiscott et al. 1999). IRF-3(5D) mimics the hyperphosphorylated and therefore activated IRF-3. The phosphorylated serine residues in activated IRF-3 are replaced by phosphomimetic aspartic acid. Transfecting cells with the IRF-3(5D) expression plasmid and the IFN-β promoter-driven reporter gene plasmid, p125-luc, should therefore lead to a strong activation of the IFN-β promoter. This was indeed observed in the experiments described in section 3.1.1.1. Here, IRF-3(5D) expression led to a 22-fold increase in IFN-β promoter activity ($p = 0.0005$). However, when either the OROV-NSs or the RVFV-NSs expression plasmid was additionally co-transfected respectively, this increase was prevented. This supports the conclusion that the OROV-NSs, similar to the RVFV-NSs (Billecocq et al. 2004), inhibits the activation of the IFN-β promoter. This result suggests that the inhibition of IFN-β promoter activation by the OROV-NSs is downstream of IRF-3 hyperphosphorylation.

The corresponding control renilla luciferase activity was however also diminished when either the OROV- or RVFV-NSs expression plasmids were respectively co-transfected (see Figure 5). It is therefore theoretically possible that the NSs proteins are cell toxic and the observed prevention of IFN-β promoter activation is indeed a pseudo one. Under the light microscope, the cells were inspected and their population had notably increased over the 24 h incubation period (between transfection and lysis). A cell toxic effect has not been described for the NSs proteins of the other Bunyaviruses. It is therefore unlikely that cell toxicity played a major role in the observed results. However, it cannot be excluded with complete certainty that

transfected cells primarily died and were replaced by non-transfected, growing cells. Further experiments should be performed to formally rule out toxicity of OROV-NSs.

The first step in trying to identify where the OROV-NSs protein prevents IFN-β promoter activation lay in determining if it inhibits the activation/expression of any of the three transcription factors (TF) that lead to IFN-β gene expression during viral infection: IRF-3, NF-κB and/or AP-1 (Hornung et al. 2006, Kato et al. 2011).

Quite a number of viruses have evolved to inhibit the IFN system by targeting a step in the activation of the TFs that bind specifically to the IFN-β promoter. For e. g. the Influenza A virus expresses the NS1 protein once inside a cell. NS1 efficiently prevents the activation of all of the three factors, IRF-3, NF-κB and AP-1 (Talon et al. 2000, Wang et al. 2000, Ludwig et al. 2002). The VP35 protein of the extremely deadly Ebola virus inhibits the activation of IRF-3 (Basler et al. 2003).

Experiments were carried out to uncover a potential inhibition: each of the TFs were respectively activated (NF-κB, AP-1) or a constitutively active mutant form (IRF-3) overexpressed in 293 cells and their TF-specific reporter gene expression plasmids were transfected into these cells together with the OROV-NSs expression plasmid. This meant that we by-pass the individual activation pathway of each of the TFs by artificially having them activated or by having a mutant constitutively active form overexpressed in the cells. The NSs should therefore lose its inhibitory effect on reporter gene expression if its mechanism of inhibition was indeed preventing the IFN-β pathway-specific activation of one of the three TFs necessary (see 3.1.1.2 to 3.1.1.4).

Co-transfection with the IRF-3(5D) expression plasmid and the IRF-3-responsive reporter gene plasmid (p55C1B-luc) showed in the absence of OROV-NSs a 166-fold increase in the firefly luciferase reporter activity which was highly significant ($p = 3.8^{-10}$). Additional transfection of the OROV-NSs expression plasmid strongly inhibited ($p = 3.8^{-10}$) this activation (see Figure 6, 3.1.1.2). That means that the OROV-NSs protein acts inhibitory even in the presence of the activated TF, IRF-3. Similar to the results described above (see 3.1.1.1), it apparently inhibits IFN expression somewhere downstream of the early steps of IRF-3 activation (phosphorylation, homodimerization). The early steps of the IRF-3 activation pathway are

therefore not significant for the inhibitory effect of NSs. Transfection with the RVFV-NSs expression plasmid served as the control since it is known that the RVFV-NSs does not inhibit IRF-3 activation, but rather works downstream of its activation (Le May et al. 2004). The results for the RVFV- and OROV-NSs proteins are similar.

The same procedure was carried out, this time artificially inducing the activation of NF-κB by adding TNF-α to the cells. Similar results were seen as with the IRF-3(5D), also indicating that the OROV-NSs protein can inhibit NF-κB-mediated promoter activation even in the presence of activated NF-κB (see 3.1.1.3). Similar results were observed, when AP-1 was artificially activated (via the MEKK pathway) in cells co-transfected with the AP-1-responsive reporter plasmid together with the OROV- and RVFV-NSs expression plasmids, respectively. The reporter gene expression was always inhibited (see 3.1.1.4). All results correlate with those of the RVFV-NSs, indicating that the OROV-NSs works in a similar way: inhibition of IFN-β promoter activation by OROV-NSs cannot be attributed to the inhibition of a specific TF.

The consistent inhibition (diminished renilla luciferase activity) of the constitutively active SV40 promoter-mediated Renilla luciferase reporter gene expression when either the OROV- and RVFV-NSs was present (see Figures 5, 7, 9, 11, 13), suggests that it is not an inhibitory process specific to the IFN-β promoter, but rather also to other promoters (even the constitutively active ones). The inhibitory effect mediated by OROV-NSs is apparently not promoter specific. This suggests that either the general gene transcription machinery of the cell could be the target of the NSs or inhibition lies at the later step of mRNA translation, or maybe a combination of both.

Both BUNV-NSs and RVFV-NSs have been shown to be inhibitors of RNAP II albeit by usage of different mechanisms (Thomas et al. 2004, Kalveram et al. 2012, Ikegami et al. 2009, Billecocq et al. 2004). It is therefore likely that OROV-NSs is also a general inhibitor of RNAP II-mediated transcription which would explain the reduction of hCMV promoter-driven Renilla luciferase reporter gene expression.

It is worth noting the potency of these NSs proteins to inhibit promoter activity: transfection with 500 ng of OROV-NSs expression plasmid resulted in the prevention of the reporter gene expression increment of factor 166 when IRF-3(5D) was present (see Figure 6). Its intense potency is also shown by the fact that only 100 ng of the transfected OROV-NSs expression

plasmid resulted in a decrement of expression of the constitutively active SV40 promoter-driven reporter gene (pRL-SV40) by factor 33 (see Figure 13). The control transfections with the RVFV-NSs expression plasmid showed even a greater potency.

4.1.2 Effects of OROV-NSs on General Gene Transcription and on Translation

The above experiments show strong inhibition of reporter gene expression by the NSs protein independent of the promoter used. This leads to the question: does the NSs inhibit cellular transcription or translation?

Experiments were carried out that skipped the cellular transcriptional process and tested the translation directly: an *in vitro* transcribed reporter RNA that expresses the firefly luciferase protein was co-transfected together with the OROV-NSs expression plasmid. The reduction of firefly luciferase protein expression would point towards a translation inhibition (since translation of RNA to protein is obviously independent of the transcription machinery).

The inhibition of gene expression of pRL-SV40 **DNA** (Figure 13, control) and pGL3 **DNA** (Figure 15, control) was compared to the inhibition of the firefly luciferase protein translation from *in vitro* transcribed RNA in the presence of the OROV-NSs (see Figures 12 and 14). When 100 ng of the OROV-NSs expression plasmid was co-transfected into cells that contained both the firefly luciferase reporter RNA and either of the DNA controls, pRL-SV40 and pGL-FFLuc, cap-independent translation of reporter RNA was not inhibited by OROV-NSs since firefly luciferase activity was reduced by only 2.3 times. However, a major inhibition of DNA-encoded luciferase activity was seen. The control renilla activity was reduced by 24 times ($p = 0.0004$) and 13 times ($p = 0.02$) for the control firefly luciferase activity. These findings point towards the inhibitory function of OROV-NSs on the transcriptional level.

Figure 12 shows that the cap-independent translation was not inhibited by OROV-NSs. However, mRNA transcribed in the cell nucleus is capped and translocated to the cytoplasm. This capped form is recognized and translated by the cellular ribosomes. It has to be considered that it is possible that the OROV-NSs inhibits only the 5’ cap-dependent translation of cellular mRNA. The *in vitro* generated RNA in the experiments described under 3.1.1.4 was uncapped, because it contained an IRES as an alternative site for translation initiation (López-Lastra et al. 2005). Therefore, the effect of OROV-NSs on cap-dependent translation was

additionally tested by usage of an *in vitro* transcribed RNA which did not contain an IRES but rather carried a cap-analog at the 5' end. Figure 14 shows the effects the OROV-NSs has on capped RNA translation (see 3.1.1.5). Similar to cap-independent translation OROV-NSs had no inhibitory effect on cap-dependent translation. It is therefore acceptable to assume that translation inhibition does not play a significant role in the pathogenicity of the OROV-NSs protein.

4.1.3 Effects of OROV-NSs on the cellular DNA-dependent RNAP II

All of these results combined suggest that it is indeed the cellular transcription that is inhibited by OROV-NSs. This assumption is supported by the observation (see Figure 16) that OROV-NSs had absolutely no inhibitory effect on the T7 promoter-driven reporter gene expression. In other words, neither the T7 RNA polymerase-mediated transcription (foreign T7 polymerase) nor translation (cellular machinery) is inhibited. The T7 system apparently has the ability to bypass the NSs inhibitory effect.

The OROV-NSs apparently has an inhibitory effect on RNAP II-dependent promoters, supporting the fact that it is the RNAP II being inhibited (maybe RNAP I and RNAP III too). The BUNV, which is of the same genus as the OROV (Orthobunyavirus), and the Phlebovirus RVFV are known to both inhibit the cellular RNAP II. However, both use different strategies (see below).

Normally, the RNAP II binds to the IFN-β gene promoter (as it does to other promoters) in the presence of basic and specific TFs. After binding of RNAP II to the promoter, the serine residues at position 5 of the 52 hepta repeats (repeat amino acid sequence: YSPTSPS) in the RNAP II C-terminal domain (CTD) are then reversibly phosphorylated. Transcription is subsequently initiated but then pauses without further modification. Elongation and the 3'-end processing of the nascent transcript requires additional phosphorylation of the serine 2 residues of the CTD-repeats and restarts the RNAP II (Kim et al. 1994, Thomas et al. 2004).

The BUNV-NSs protein prevents this elongation and/or 3'-end processing of the IFN-β mRNA by inhibiting serine 2 phosphorylation of the RNAP II CTD (Thomas et al. 2004). This reduces overall gene transcription. The BUNV-NSs inhibits the cells RNAP II transcriptional activity by interacting with the protein MED8 (Léonard et al. 2006), which is a subunit of the mediator

complex that interacts with the CTD of RNAP II in its hypophosphorylated form. The complex activates CTD phosphorylation using the TFIIH kinase (Kim et al. 1994, Kobor and Greenblatt 2002). With such a close relative of the OROV using this strategy, it was of course critical to determine if the OROV-NSs exhibits the same mechanism of transcription inhibition. The RNAP II phosphorylation state at the serine 2 sites of the repeats would have to be looked at, when OROV-NSs was present. Notably, the domain of the BUNV-NSs protein that interacts with the MED8 complex contains an amino acid motif that is highly conserved amongst the NSs proteins of viruses of the Bunyamwera and California serogroups (Léonard et al. 2006). There is currently no data comparing these domains with that of the Simbu serogroup.

Even though the NSs protein of the Phlebovirus RVFV has hardly any sequence similarity to that of the BUNV-NSs (Billecocq et al. 2004), it has been shown to also prevent the phosphorylation of serine 2 on the repeats (unpublished data by Dr. rer. nat. Martin Spiegel, Virology Institute, Göttingen). This prevents elongation and/or 3'-end processing of the IFN-β gene transcript.

Interestingly, the RVFV-NSs employs a different mechanism in preventing the serine 2 phosphorylation. Unlike the BUNV-NSs, it directly targets the TFIIH kinase by interacting with its p44 subunit. This interaction prevents the assembly of the enzyme subunits to build a functional unit. The filamentous structures seen in the cell nucleus of cells transfected with the RVFV-NSs expression plasmid (see Figures 17 to 19) comprise of RVFV-NSs and some subunits of the TFIIH kinase including p44 (Le May et al. 2004). Such structures are not seen in the cell nucleus of the cells expressing the OROV-NSs.

Experiments were carried out to look at the phosphorylation state of the RNAP II in the presence of NSs (see 3.1.2). The phosphorylation of serine 5 (initiation of transcription) and of serine 2 (elongation of transcript) of the hepta repeats of the CTD were looked at by incubating the cells with antibodies against the respective phosphorylated sites.

The antibodies against phospho-serine 5 found their target in cells transfected with the OROV-NSs expression plasmid (see Figure 18): there was no reduction in the phosphorylation of serine 5 meaning initiation of transcription by RNAP II was not affected. Interestingly, there was also no weakening of fluorescence signal in the presence of OROV-NSs when antibodies against phosphor-serine 2 were used, in contrast to the results obtained for the BUNV- and

RVFV-NSs (see above). The RNAP II is therefore “switched on” by the cell and it should be able to initiate transcription and elongate the transcript. Close inspection of the confocal microscope images revealed, that most of the cells that express the OROV-NSs have a very distinct distribution of the RNAP II in comparison to the control. When OROV-NSs is present, active RNAP II (= phosphorylated at serine 2 and 5, Figures 17 and 18) seems to reorganize itself in the cell nucleus into regions of high polymerase amount. In the control, the active RNAP II is distributed over the entire cell nucleus rather equally (see Figure 19). This RNAP II reorganization also appears to only involve the active RNAP II (H5 and H14), since the regions of high fluorescence are not seen with the N20 antibody that targets both active and inactive RNAP II (see Figure 19). Some OROV-NSs protein positive cells however do not show this cluster phenomenon. This could mean that such an aggregation of active RNAP II is time (phase) dependent. On Figure 19, there is no weakening of the N20 signal to be seen in the presence of the OROV-NSs, suggesting that there is no RNAP II degradation induced by OROV-NSs (fluorescence signal similar to that of the control).This means that the reduction in transcriptional activity induced by the OROV-NSs is not due to degradation of RNAP II.

On close inspection, the OROV-NSs seems to form circular ring-like structures in the cell nucleus in contrast to the filamentous structures formed when the RVFV-NSs protein associates with the p44 subunit of the TFIIH kinase.

All results taken together, the OROV-NSs protein seems to inhibit transcriptional activity using another strategy other than that of its relatives BUNV and RVFV.

It is interesting that the OROV, RVFV and the BUNV would all inhibit general gene transcription of their host cells, because they themselves are directly dependent on the host cells mRNAs (Bouloy et al. 2001). The viral L proteins cut capped oligonucleotides off the 5’ end of the host cells mRNAs and the virus uses them as its own primers – this is an obligatory process for viral transcription (Elliott and Weber 2009). It is therefore interesting, why they inhibit general gene transcription, as this would seize their supply of new cellular mRNA. The close relative to the OROV, the LACV, has been examined from this perspective and it turns out that inhibiting the RNAP II with actinomycin D, does not suppress viral RNA synthesis (Raju and Kolakofsky 1988). Apparently, full RNAP II activity is not that crucial for the viruses and the cytoplasmic pool of cellular mRNAs is sufficient for viral RNA synthesis and production of progeny viruses.

The general suppression of host cell gene expression is probably not only based on the activities of the NSs proteins, but also on the fact that the 5' end, including the cap, of cellular mRNAs are stolen by the viral L protein. Without such a protective cap end, the cellular mRNAs are degraded by proteasomes within the cell (Raju and Kolakofsky 1988).

Furthermore, inhibition of gene expression by Orthobunyaviruses is not observed in infected vector insect cells which seem to be persistently infected (Hacker et al. 1989), suggesting a specificity of inhibition to mammalian cells. The difference between insect and mammalian CTD of the RNAP II are 10 amino acids conserved only among vertebrates. These 10 amino acids have been found to be important in conjunction with the phosphorylated serines 5 and 2 of the 52 repeats for efficient transcription (Fong et al. 2003).

4.2 The OROV Minireplicon System

Minireplicons are very important tools for understanding viral transcription and replication.They allow studying these processes in the absence of infectious virus. They consist of expression plasmids for the viral polymerase and the viral nucleoprotein and a minigenome. The minigenome mimicks a viral genomic segment (reporter gene flanked by viral NTRs) and can only be transcribed by the viral polymerase. Recently, an attempt was made to establish a minireplicon for OROV (Keisers). This system was however inactive: transfection of BSR-T7 cells with the OROV minigenome plasmid together with the OROV nucleocapsid (N) and polymerase (L) expression plasmids did not lead to the expected renilla luciferase gene expression (see Figure 20 and 22). The aim here was to find the cause of the problem.

Firstly, possible procedural errors had to be excluded by creating a LACV minireplicon parallel to that of the OROV. The LACV minigenome, -N and –L protein expression plasmids were transfected exactly the same way the OROV expression plasmids were. The LACV minireplicon system resulted in high renilla luciferase activity (see Figure 20 and 22). Therefore, the problem was apparently not a methodical one.

Experiments were then carried out to indirectly examine the functionality of the OROV minigenome. For this, the LACV-L and –N protein expression plasmids were co-transfected together with the OROV minigenome expression plasmid (see Figure 20/B). The LACV polymerase (L) and nucleoprotein (N) apparently recognized the OROV minigenome. The

renilla luciferase activity was a lot higher than that of the negative control (although not as high as when only LACV components were present; see Figure 20/A). The NTRs of Orthobunyaviruses being quite conserved could at least in part explain the recognition of the OROV minigenome by the LACV components.

The OROV-L and –N protein expression plasmids were then both individually examined for functionality by co-transfecting them respectively with the other components of the LACV. Transfection with the OROV-L and/or –N protein expression plasmids together with components of the LACV minireplicon (see Figure 22) was unable to induce significant renilla luciferase gene expression.

It was therefore assumed that either the OROV-L or –N was dysfunctional. The OROV-N and –L expression plasmids were sequenced in search of mutations. The N expression plasmid sequence was correct. There were however six mutations (see 3.2) in the OROV polymerase gene (L), when compared to the L segment sequence of the OROV in the NCBI gene bank. These six mutations (insertions) would mean the addition of two amino acids in the L protein, which could very well lead to a dysfunctional OROV polymerase. This mutated sequence of the OROV polymerase gene was then compared to the sequences of the other Orthobunyaviruses. It turned out that the other Orthobunyaviruses have the same mutated sequence. It appears to be a highly conserved sequence amongst the Orthobunyaviruses (Keisers). The published reference sequence for the OROV polymerase is therefore probably wrong. Hence, it is possible that these mutations have no negative influence on the functionality of the OROV polymerase.

Each segment of the OROV genome has a promoter region that is not translated (NTR). The 5’ NTR of a particular segment is partially complementary to the 3’ NTR of the same segment. That is why each of the three OROV segment tend to form a panhandle structure. These regions were initially not sequenced during the research of this thesis.

The ORF of the OROV minigenome was sequenced and found to be correct. After conclusion of this thesis, it was found that both NTR sequences of the minireplicon were correct in comparison to the international gene bank. The published NTR sequence however differs from those of the other Orthobunyviruses in one nucleotide, although this region is otherwise highly conserved (Keisers). This different nucleotide would form an additional bond eventually

affecting the activity of the viral polymerase. The reference sequence used to create the OROV minigenome could therefore be wrong. Therefore, the sequence of the NTRs has to be experimentally confirmed. A possible approach involves isolation of viral RNA from virus particles instead of infected cells. In contrast to RNA from cells, which represent a mixture of viral and cellular mRNAs and viral genome segments, RNA from viral particles contains only genome segments. Subsequent 5’ and 3’ RACE-RT-PCR using this viral genomic RNA will recover the NTRs, which can then be sequenced.

Getting this minireplicon system to work is empirical. An interesting breakthrough in the prevention of Bunyaviridae infections using a reverse genetics system has been described for the LACV (Blakqori and Weber 2005). The LACV was completely artificially assembled. For this, the M segment of the virus was also cloned and transfected into a cell line in addition to the other minireplicon components (see above). This recombinant LACV was based on cDNA that was mutated on the S segment and expressed the N protein but not the NSs protein. When mice were infected with this recombinant virus that lacks its major virulence factor, they generated a protective immunity strong enough to prevent the infection by the wild type LACV.

5 Summary

The OROV is a RNA virus that has a segmented, single stranded genome of negative polarity. It belongs to the *Bunyaviridae* family. Infection by the OROV causes an acute very debilitating febrile episode. This RNA virus is responsible for multiple and massive epidemic outbreaks in South America involving hundreds of thousands of infected people.

The viruses of this family express a NSs protein as their major virulence factor, which inhibits the expression of IFN-β. IFN-β is a critical antiviral protein, whose expression, if inhibited, allows for the replication of virus particles. The experiments carried out in this thesis aim at characterizing the pathogenic mechanism of the NSs protein of the OROV.

There are three transcriptional factors that are necessary for IFN-β gene expression and they are activated after viral recognition: IRF-3, NF-κB and AP-1.

The OROV-NSs protein blocks the activation of the IFN-β promoter as well as the activation of the artificial IRF-3-, NF-κB- and AP-1-responsive promoters, and the constitutively active SV40 promoter. The inhibitory effect of the OROV-NSs does not lie in translation inhibition since mRNA can still be translated to protein in the presence of the NSs protein, irrespective of the cap-/IRES-status of the mRNA. Instead, the OROV-NSs inhibits IFN-β gene transcription probably by inhibiton of the RNAP II. A T7 polymerase based expression system can bypass the NSs inhibitory effect, showing certain specificity for inhibition of transcription mediated by mammalian RNAP II.

In contrast to the NSs of its close relatives, BUNV and RVFV, the OROV-NSs does not influence the phosphorylation of the serine 2 (elongation and 3'-end processing) of the heptapeptide repeat at the CTD of the RNAP II. The OROV-NSs also does not prevent the phosphorylation of serine 5. Its transcription inhibitory effect is not due to the induction of degradation of the RNAP II.

The OROV-NSs induces a reorganization of active RNAP II in the nucleus of some cells and is probably associated with the RNAP II-containing transcription complex.

The second part of this thesis involved setting up a functional minireplicon system for the OROV, as this has never been done before. A minireplicon system is critical in understanding a viruses transcription and replication processes. Experiments carried out, showed that the

functional OROV minireplicon system could not be established probably due to sequence errors either in the OROV-L ORF or in the NTRs of the OROV minigenome, which were present in the expression constructs due to the faulty OROV reference sequences in the international gene bank (NCBI).

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Curriculum Vitae:

I was born on the 16th of October 1986 in Ikot Ekpene, Nigeria, to my mother, Monika Essien, who is a German social worker in Nigeria running a primary school and to my father, Anthony Essien, who is the director of a secondary school in Nigeria.

I attended my primary school at Monika Kindergarten, Ikot Ekpene, Nigeria, from 1989 to 1996. After that, I shortly attended a Federal Government College in Ikot Ekpene, Nigeria from 1996 to 1999. Due to its poor educational standard, I changed to the catholic school St. Paul Academy in Ikot Ekpene, which I attended from 1996 to 2003. I wrote my O-level examinations there.

From 2003 to 2004 I attended a German language school in Hamburg, Germany, and from 2004 to December 2005, I attended the Studienkolleg in Hamburg, where I also wrote my German A-level examination (= Abitur).

In March 2006, I started studying human medicine at the Georg-August-University of Göttingen. In March 2008, I wrote the "1. Abschnitt der ärztlichen Prüfung" and began, with scholarship sponsorships by the Reemtsma Begabtenstiftung and then the Heinrich-Böll-Stiftung, with my clinical studies. I successfully ended my medical studies with an overall score of „Sehr Gut" in May, 2012.

From December of 2012 to July, 2014 I worked in DIAKO, Bremen, in the department of General, Thorax, Adipositas and Vascular Surgery. Since August 2014 I have been working on the surgical intensive unit at the university clinic of Lübeck.