

**Interaction of African horse sickness virus inclusion bodies with
cellular translation machinery to compartmentalise virus protein
synthesis**

by

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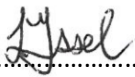
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Under the supervision of

Dr Vida van Staden and Dr Eudri Venter

Declaration

I, **Litia Yssel** declare that the thesis/dissertation, which I hereby submit for the degree **Magister Scientiae** at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature:

Date: 17 November 2019

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Summary

Interaction of African horse sickness virus inclusion bodies with cellular translation machinery to compartmentalise virus protein synthesis

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African horse sickness virus (AHSV) is an orbivirus in the *Reoviridae* family that causes severe disease in horses, with major economic implications. The viral genome consists of ten double-stranded RNA segments, encoding seven structural viral proteins (VP) plus four non-structural (NS) proteins. The non-structural proteins serve to enhance the viral life cycle by influencing viral morphogenesis, replication or egress. In orbiviruses, NS2 multimers form dense cytoplasmic matrices termed viral inclusion bodies (VIBs). It has until recently been assumed that the VIBs of AHSV merely serve as replication factories and sites wherein virus particle assembly occurs. However, various different viruses have been shown to compartmentalise virus protein synthesis within cytoplasmic replication factories, which is believed to contribute to enhancing the viral life cycle. Little is known about how AHSV utilises the host translational machinery for its replicative advantage.

In this study, the distribution and morphology of AHSV VIBs were characterised at different stages of the replication cycle. The VIBs were shown to increase both in size and abundance up until a certain time point

after infection. The formation of the VIBs from NS2 precursors was characterised by making use of plasmid-expressed fluorescent NS2-eGFP. Preliminary results from live-cell imaging revealed the formation of the VIBs to be a dynamic process, involving the progressive coalescence of small NS2 foci in a random fashion.

Using a biochemical assay followed by confocal microscopy analyses, it was investigated whether the VIBs are sites of protein synthesis. Our results showed that active translation was substantially enhanced within the VIBs of AHSV-4, AHSV-5 and BTV-10, surpassing the intensities of cytoplasmic protein synthesis. This indicates virus-mediated compartmentalisation of translation within the VIBs as a broad functionality amongst the orbiviruses.

We next determined whether different eukaryotic ribosomal components and translation initiation factors localise to the VIBs of AHSV. Interestingly, we observed differential distribution patterns of these components, indicating distinct sub-structural domains of the VIBs. The ribosomal component L11 and initiation factor eIF30 localised throughout the VIBs, with elevated intensities towards the central regions, whereas ribosomal component S3 and initiation factor eIF4E localised to the VIB perimeters. These localisation patterns were shown to be solely mediated by NS2.

These results indicate a novel functionality of the VIBs, by serving as hubs for virus protein synthesis. It still remains to be elucidated how translation is orchestrated within the VIBs, however our preliminary results could indicate to the potential for sub-domains of the VIBs being involved in specific functions regarding translation. Overall, this indication of translation compartmentalisation within the VIBs could elucidate a strategy whereby AHSV enhances the viral infection cycle.

Abbreviations

°C	degrees Celsius
AHSV	African horse sickness virus
µg	micrograms
µl	microlitres
µm	micrometers
A.F.	Alexa Fluor-conjugated secondary antibody
aa	amino acids
BacNS2	recombinant baculovirus expressing NS2
bp	base pairs
BSR-T7	baby hamster kidney cells with T7 promoter
BTV	bluetongue virus
CLSM	confocal laser scanning microscopy
CPE	cytopathic effect
DAPI	4',6-diamidino-2-phenylindole
ddH ₂ O	double distilled water
DNA	deoxyribonucleic acid
ds	double stranded
eGFP	enhanced green fluorescent protein
eIF	eukaryotic translation initiation factor
EtBr	ethidium bromide
EtOH	ethanol
G	gauge
h	hours
h p.i.	hours post-infection
h p.t.	hours post-transfection
kDa	kilodaltons
LB	Luria-Bertani
min	minutes
MeAc	methanol-acetone
MEM	Minimal essential medium
MeOH	methanol
MIP	maximum intensity projection

MOI	multiplicity of infection
MP	milk powder
nm	nanometers
NS2	non-structural protein 2
O/N	overnight
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
phCMV-NS2	transient expression vector with phCMV promoter expressing NS2
phCMV-NS2-eGFP	transient expression vector with phCMV promoter expressing NS2-eGFP
PMY	puromycin
PSB	protein solvent buffer
rcf	relative centrifugal force
RE	restriction endonuclease
RNA	ribonucleic acid
rpm	revolutions per minute
RPM	ribopuromycylation
RT	room temperature
SDS	sodium dodecyl sulphate
Seg	segment
Sf9	<i>Spodoptera frugiperda</i>
ss	single stranded
TAE	tris-acetate-EDTA buffer
TCID ₅₀	50% tissue culture infectious dose
TGS	tris-glycine-SDS buffer
VF	viral factory
VIB	viral inclusion body
w/v	weight per volume

List of buffers

Hypotonic lysis buffer	0.5% Triton-X, 10 mM Tris-HCl pH 7.4, 1 mM EDTA pH 8, 150 mM NaCl
IP buffer	150 mM Tris-HCL, 50 mM NaCl, 5 mM MgCl ₂ , 1% Triton-X, supplemented with 10 U/ml RNase inhibitor, 1 x proteinase-and-phosphatase inhibitor, 25 U/ml Benzonase nuclease
LB medium	1% tryptone, 0.5% yeast extract, 1% NaCl
Permeabilisation buffer	50 mM Tris-HCl [pH 7.5], 25 mM KCl, 5 mM MgCl ₂ , 355 µM cycloheximide, 5 U/µl Pepstatin, 10 U/ml RNase inhibitor, and 0.2 % Triton X-100, supplemented with 4% PFA
1 x PBS	137 mM NaCl, 2.7 mM KCl, 4.3 mM Na ₂ HPO ₄ ·2H ₂ O, 1.4 mM KH ₂ PO ₄ , pH 7.4
6 x PSB	0.188 M Tris pH 6.8, 6% SDS, 30% Glycerol, 15% 2-Mercaptoethanol
1 x TAE	40 mM Tris, 20 mM acetic acid, 1 mM EDTA
1 x TGS	2.5 mM Tris, 19.2 mM glycine, 1% SDS
Townbin's transfer buffer	25 mM Tris, 192 mM glycine, 20% methanol
wash buffer	0.05% Tween-20 in 1 x PBS

Chapter 1

Literature Review

1.1. Introduction

Viruses are dependent upon the host translation machinery to synthesise viral proteins, as these are not encoded for by viral genomes. Different viral strategies have been adapted for taking ownership of cellular translational components to enhance the viral infection cycle. African horse sickness virus (AHSV) is an arthropod-borne virus endemic to Africa. The virus is transmitted by *Culicoides* midges and infected horses develop African horse sickness (AHS), a serious disease with mostly fatal outcomes. AHSV is a double-stranded RNA (dsRNA) virus and a member of the *Orbivirus* genus within the *Reoviridae* family. Shortly after an infection with AHSV, the viral non-structural protein NS2 assembles to form dense granular cytoplasmic aggregates termed viral inclusion bodies (VIBs) (Brookes *et al.* 1993; Uitenweerde *et al.* 1995). It has long been assumed that AHSV translation is restricted to the cytosol, and that the VIBs merely serve as sites for viral replication and assembly of progeny virions (Kar *et al.* 2007). The strategies employed by AHSV to take over components of the host translation apparatus are not yet fully understood.

Numerous different viruses have been shown to compartmentalise viral translation within their cytoplasmic replication factories that have core functionalities similar to the AHSV VIBs (Katsafanas and Moss 2007; Sánchez *et al.* 2013; Desmet *et al.* 2014; Schoelz and Leisner 2017). Among these is mammalian orthoreovirus, also a member of the family *Reoviridae*, which was shown to sequester host protein synthesis machinery and actively translating ribosomes within their viral factories (Desmet *et al.* 2014). This raised the question as to whether the VIBs of AHSV have an additional function by becoming sites for virus protein synthesis. This formed the main focus of this study, and could elucidate a strategy whereby AHSV enhances the viral life cycle. As background to this investigation, this literature review will cover the molecular biology of the orbiviruses, and compare the components and functions of orbivirus VIBs with cytoplasmic replication factories formed by other viruses. Different strategies whereby viruses up-regulate viral protein synthesis at the cost of host translation, including what is known about orbivirus translation regulation, will be discussed.

1.2. History of African horse sickness

African horse sickness (AHS) was identified for the first time in 1719 on the African continent when it caused the death of 1700 animals (reviewed by Long and Guthrie 2007; Roy 2007). Another severe outbreak occurred in 1854 in South Africa, affecting approximately 70 000 horses. It was discovered in 1900 that AHS is caused by a viral infection (M'Fadyean 1900), and the existence of multiple serotypes for AHSV was discovered shortly thereafter (Theiler 1908). The first polyvalent vaccines were developed in the 1930s (Alexander and Du Toit 1934). The vector of AHS, the *Culicoides* midge, was described in 1944 (Du Toit 1944). Although

endemic to Africa, epidemic AHS outbreaks have been recorded in India, Pakistan, Turkey, Israel, Iran and Palestine (reviewed by Roy 2007).

1.3. African horse sickness pathology

The clinical outcomes of AHS ranges from mild to severe, the prognosis depending on the species affected and the host adaptive immune system (reviewed by Mellor and Mertens 2008; Long and Guthrie 2014). Horses are the most susceptible species, while donkeys and zebras most often do not present with any symptoms, and are thus reservoirs for AHSV. As the disease is transmitted by midges, its spread is highly influenced by climatic conditions. After a horse had been bitten by an infected midge, infection initiates in the lymph nodes, and spreads to the endothelial cells of target organs, mainly the spleen, lungs and heart (Gomez-Villamandos *et al.* 1999). There are three distinct forms of the disease plus a mixed form, which differ in severity. The three main types are the pulmonary form, cardiac form, and horse sickness fever. The pulmonary form is the most severe form of AHS, and often entails respiratory failure and sudden death. The cardiac form is the second most severe, and entails damage to the heart muscles accompanied by swelling of the supraorbital fossae or the entire head (Maurer and McCully 1963). The mixed form is the most common form of AHS, and entails a combination of the pulmonary and the cardiac form (Carpano 1931). Horse sickness fever is the least severe, and most frequently occurs in immune horses or non-susceptible species.

1.4. Measures to diagnose and control African horse sickness

There are currently no treatments available for AHS, and thus efforts go into preventing the emergence of the disease. During the past twenty years, real-time reverse transcription polymerase chain reaction (RT-PCR) assays have been developed to diagnose AHSV infection (reviewed by Dennis *et al.* 2019). These include group-specific tests, directed at recognising the gene encoding a single structural and/or non-structural protein of AHSV (Rodriguez-Sanchez *et al.* 2008; Romero and Jime 2008; Quan *et al.* 2010; Guthrie *et al.* 2013; Bachanek-Bankowska *et al.* 2014), two of which have been approved by the World Organisation for Animal Health as official screening tests. However, the most refined diagnostic procedures allow for the detection of three serotypes in a single assay (Weyer *et al.* 2015). Preventative measures are being executed in the form of vaccination. The current polyvalent live-attenuated vaccines contain all but serotypes 5 and 9 of AHSV (Teichman and Smit 2008), as these serotypes have been found to be cross-protected against by other specific serotypes within the vaccine (Teichman *et al.* 2010). Reverse genetics systems have been developed for AHSV, and are ongoingly investigated for their potential towards the development of attenuated vaccines (Boyce *et al.* 2008; Matsuo *et al.* 2010; Kaname *et al.* 2013; Water *et al.* 2015; Conradie *et al.* 2016; Lulla *et al.* 2016, 2017; van Rijn *et al.* 2016; van Rijn *et al.* 2018).

1.5. Orbivirus replication cycle and molecular biology

The morphology and replication processes of AHSV share many similarities with bluetongue virus (BTV), a related ruminant-infecting orbivirus (Roy *et al.* 1994). BTV has been studied to a significantly greater extent, and sometimes serves as a prototype for AHSV (Patel and Roy 2014). Nine distinct serotypes exist for AHSV (AHSV-1 to AHSV-9) (McIntosh 1958; Howell 1962). Reverse genetics systems have recently been developed for both AHSV and BTV (Boyce *et al.* 2008; Matsuo *et al.* 2010; Kaname *et al.* 2013; Vermaak *et al.* 2015; Conradie *et al.* 2016; van Rijn *et al.* 2016). These platforms have enabled the identification of essential components of the virus life cycle and assigning of functional roles to genomic segments and protein domains. The virus particle is non-enveloped and its genome consists of ten double-stranded RNA (dsRNA) segments (Bremer 1976). The viral genome is enclosed by a triple layer of structural viral proteins (VPs), with VP3 and VP7 forming the two layers of the core, and VP2 and VP5 forming the outer capsid (Manole *et al.* 2012). Enclosed within the core is a transcriptase complex (TC) consisting of the proteins VP1, VP4 and VP6 which drives viral replication and transcription (Ramadevi *et al.* 1998; De Waal and Huismans 2005; Mertens *et al.* 2009). In addition to the structural proteins, the viral genome encodes non-structural (NS) proteins namely NS1, NS2, NS3 and NS4 which play distinct roles in either viral replication, morphogenesis or viral egress (Grubman and Lewis 1992; Kar *et al.* 2007; Boyce *et al.* 2012; Zwart *et al.* 2015; Ferreira-Venter *et al.* 2019).

1.5.1. Orbivirus replication cycle

An overview of the orbivirus replication cycle is depicted in Fig. 1.1 (Dennis *et al.* 2019). BTV is known to attach to a glycoprotein receptor on the cell surface, followed by entry via clathrin-mediated endocytosis (Forzan *et al.* 2007). AHSV-4 has also been shown to enter certain cell types via macropinocytosis (Vermaak *et al.* 2016). The low pH within the endosome causes the outer capsid proteins to disassemble, and the resulting core particle exits the endosome into the cytoplasm (Forzan *et al.* 2007). Within the core, positive-sense RNAs (+RNAs) are transcribed from negative-sense RNA (-RNA) templates. The transcripts obtain capped structures at the 5' end (Ramadevi *et al.* 1998) and exit the core into the cytoplasm, where they serve as templates for translation or genomic dsRNA synthesis. Utilising the host translational machinery, the ten unique viral +RNA segments are translated to produce viral structural and non-structural proteins, including NS2. Oligomers of NS2 form dense cytoplasmic structures known as viral inclusion bodies (VIBs) (Brookes *et al.* 1993; Uitenweerde *et al.* 1995). The VIBs of BTV recruit individual viral ssRNA segments (Lympieropoulos *et al.* 2003), core proteins VP3 and VP7 and the TC components VP1, VP4 and VP6 (Modrof *et al.* 2005; Kar *et al.* 2007), where complete genomic sets are packaged into the new core particles. Inside the core particle, the +RNAs serve as templates for replication of -RNAs to form the dsRNA genome segments. The newly assembled cores are extruded from the VIBs and ultimately acquire the outer capsid proteins VP2 and VP5

at a yet unknown location in the cytoplasm, forming mature progeny virus particles. Finally, the virus exits the host cell either by cell lysis or budding-like egress (Stoltz *et al.* 1996).

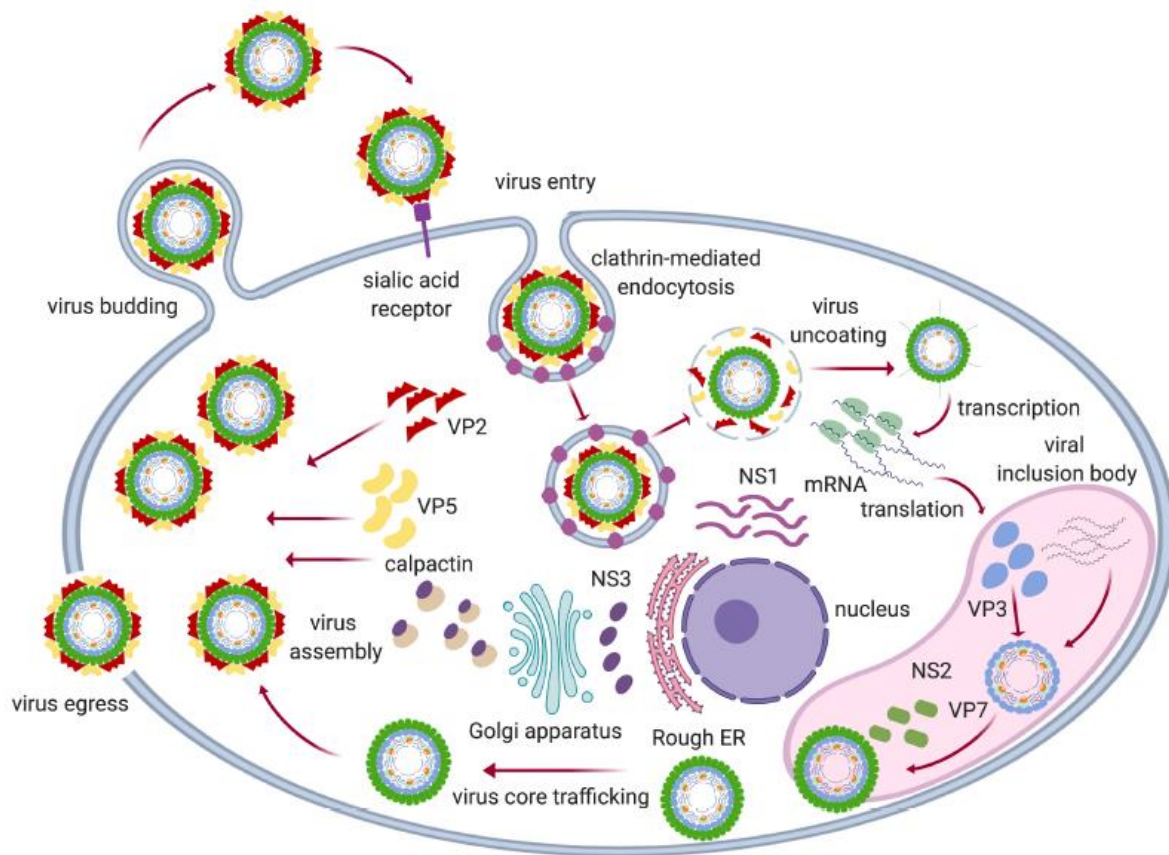


Fig. 1.1: An overview of the orbivirus replication cycle, from Dennis *et al.* (2019). (Copyright permission for use of this diagram was obtained from *Viruses*).

1.5.2. Virus molecular biology

1.5.2.1. The genome

The genome of AHSV is comprised of ten dsRNA segments (segment 1 (Seg-1) to segment 10 (Seg-10)), with sizes ranging from around 3200 base pairs (bp) for the largest Seg-1 to 756 bp for Seg-10 (Bremer 1976; Bremer *et al.* 1990). All but two of the RNA segments encode a single protein, the exceptions being Seg-9, which encodes both VP6 and NS4 from two different reading frames (Zwart *et al.* 2015), and Seg-10, which encodes both NS3 and the shorter NS3A from the same reading frame (van Staden and Huismans 1991). In earlier years, only eight of the ten genomic segments of AHSV were sequenced (van Staden and Huismans 1991; van Staden *et al.* 1991; Roy *et al.* 1991; Mizukoshi *et al.* 1992; Iwata *et al.* 1992; Mizukoshi *et al.* 1993). These findings revealed the GC content of the viral genome to be 43%, with the 3' non-coding regions being longer than the 5' regions (reviewed by Roy *et al.* 1994). Different from BTV, the non-coding regions of different AHSV serotypes do not show full sequence conservation across all segments, however some

sequences are generally similar, with the 5' non-coding region starting with 5' GUUAAA.... 3' and the 3' non-coding region ending in 5' ACUUAC 3'. The different segments form secondary structures, due to regions of inverted sequence complementarity in the 5' and 3' non-coding regions (Roy *et al.* 1991). The secondary structure formation might serve as protection of the viral mRNA against digestion by host exonucleases, and have been shown to be essential for BTV genome packaging (Burkhardt *et al.* 2014). In the case of BTV, viral genomic sets have been shown to interact with one another in synchronised steps and form complexes prior to their assembly within core structures (Sung and Roy 2014).

1.5.2.2. *The transcriptase complex*

AHSV replication and transcription is driven by the viral transcriptase complex (TC), which is comprised of the viral structural proteins VP1, VP4 and VP6. VP1 of AHSV is encoded by Seg-1 of the viral genome and possesses RNA-dependent RNA polymerase activities (Boyce *et al.* 2004). In the presence of magnesium, VP1 incorporates nucleoside triphosphates (NTPs) present in the core to synthesise novel RNA strands (Mertens *et al.* 2009). VP4 is encoded by Seg-4 of the viral genome and serves as a capping enzyme, which confers an essential post-transcriptional modification to the viral RNAs (Ramadevi *et al.* 1998). Through its nucleotide phosphorylase, guanylyl transferase and trans methylase activities, VP4 adds a 7-methylguanosine cap to the 5' terminus of newly synthesized strands of viral +RNA. The capping of the viral RNA by VP4 occurs in two steps. First, a methyl is transferred to 7' guanine, forming m⁷GpppG. . . followed by the transfer of a second methyl, yielding the m⁷GpppGm cap. VP6 is encoded by Seg-9 of the viral genome, and functions as an RNA helicase (De Waal and Huismans 2005). Through adenosine triphosphate (ATP) hydrolysis, VP6 unwinds viral dsRNA prior to transcription and translation. The affinity of VP6 for dsRNA has been shown to be largely affected by pH and salt concentration. The RNA binding regions of VP6 have been mapped, and the RNA interactions have been shown to be essential for virus genome packaging and replication (Sung *et al.* 2019).

1.5.2.3. *The core*

The core particle of AHSV is a double-layered structure, with VP3 and VP7 forming the inner and outer core layers respectively (Manole *et al.* 2012). VP3 proteins provide scaffolding for VP7 proteins towards the capsid face, as well as for the TC proteins towards the inner layer of the core particle. The core encloses the viral genomic segments, shielding them from recognition by host defences. VP3 is a hydrophobic protein of 103 kDa in size and is encoded by Seg-3 of the viral genome. Shallow grooves present between VP3 oligomers allow for the release of the viral RNAs from the cores to the cytoplasm (Grimes *et al.* 1998). VP7 is encoded by Seg-7 of the viral genome and is 38 kDa in size (Roy *et al.* 1991). While BTV VP7 has been shown to stabilise the transcriptase complex, it is not required for primary replication, however VP3 is essential for this process (Matsuo and Roy 2013). In addition to assembling into the core particle, VP7 trimers of AHSV have been

shown to also form large crystalline-like cytoplasmic structures in infected cells of yet unknown functions (Bekker *et al.* 2014).

1.5.2.4. *The capsid*

The third and outermost layer of the AHSV particle is the capsid, which is comprised of the viral structural proteins VP2 and VP5 (Manole *et al.* 2012). VP2 is encoded by Seg-2 of the viral genome and is 124 kDa in size. It is a hydrophobic protein which forms trimers of which there are 60 triskelions on the viral surface. VP2 is the most variable AHSV protein and specifies the serotype of the virus (Potgieter *et al.* 2003). It recognises and binds to glycoprotein receptors on the host cell surface, thereby facilitating viral entry, and has also been shown to be a target for neutralisation by host antibodies (Burrage *et al.* 1993). VP5 is encoded by Seg-6 of the viral genome and is 57 kDa in size. It possesses membrane destabilising activities and thus aids in cell permeability during viral entry (Stassen *et al.* 2011). In the case of BTV, VP5 has also been shown to facilitate the release of viral cores from the endosome in a pH-dependent manner (Eaton and Brookes 1990).

1.5.2.5. *The non-structural proteins*

In addition to the proteins that comprise the structure of the virus, the viral genome encodes four non-structural proteins that play key roles in enhancing the viral replication cycle.

NS1 is encoded by Seg-5 of the viral genome and is 63 kDa in size (Mizukoshi *et al.* 1992). It shows high sequence conservation among different orbiviruses. Electron microscopic studies revealed NS1 of AHSV to form tubule structures (Huismans and Els 1979), the morphological characteristics of which were later characterised in more detail (Maree and Huismans 1997). NS1 has been shown to enhance BTV translation on RNA level, however the exact mechanism by which this occurs remains unknown (Boyce *et al.* 2012). The atomic structure of NS1 has been determined (Kerviel *et al.* 2019). The non-tubular conformation of NS1 has been shown to also enhance BTV replication and RNA translation, and it has been revealed that these functions require the presence of specific zinc-finger motifs (Kerviel *et al.* 2019).

NS2 is encoded by Seg-8 of the viral genome and is 41 kDa in size. Due to its many charged residues, NS2 is hydrophilic in nature. It is the only phosphorylated orbivirus protein and has ssRNA binding affinities (Huismans *et al.* 1987). Shortly after infection, NS2 oligomerises and forms large matrixes in the cytoplasm, termed viral inclusion bodies (VIBs) (Brookes *et al.* 1993), which can form in the absence of other viral proteins (Uitenweerde *et al.* 1995). In BTV-infected cells, the VIBs have been characterised as providing sites for the replication of the viral genome and assembly of the genomic segments into core particles (Kar *et al.* 2007). More details about the VIBs will be discussed in later sections.

NS3 and NS3A are encoded by two in-frame start codons of Seg-10 of the viral genome and are approximately 25 kDa in size. NS3A is a truncated version of the NS3 protein, with a yet unknown function. NS3 and NS3A are integral membrane proteins that play essential roles in virus release by altering the permeability of the cell membrane (Meiring *et al.* 2009). It has been shown that NS3 interacts with VP2 and the cellular protein p11 (Beaton *et al.* 2002). The different concentrations of NS3 in different host cells, for example mammalian vs. insect cells, are suggested to account for the different modes of viral egress. NS3 of BTV was found to be essential for intracellular virus trafficking (Celma and Roy 2011) and to interfere with the host immune response (Chauveau *et al.* 2013). In the case of AHSV, the C-terminal region, as well as both the transmembrane motifs and late domain motifs of NS3 and NS3A were shown to be essential for virus trafficking and release (Ferreira-Venter *et al.* 2019). However, mutation of the start codon of NS3A does not significantly affect virus egress (Ferreira-Venter *et al.* 2019).

NS4 is encoded by Seg-9 of the viral genome and has only recently been characterised for BTV (Belhouchet *et al.* 2011; Ratinier *et al.* 2011) and for AHSV (Zwart *et al.* 2015). Two different forms of AHSV NS4 with slightly different amino acid sequences, namely NS4-I and NS4-II, are encoded by different viral serotypes, and are approximately 17 and 20 kDa in size respectively (Zwart *et al.* 2015). Both types of AHSV NS4 have been shown to bind dsDNA (Zwart *et al.* 2015), and BTV NS4 plays an essential role in suppressing host interferon responses (Ratinier *et al.* 2016). The exact functional role of NS4 in AHSV replication however remains to be determined.

1.6. Viral inclusion bodies and related structures

The formation of cytoplasmic viral replication factories is an integral part of various viruses' infection cycles. The main function of the replication factories is to serve as the sites for viral genome replication and/or virus particle assembly. The replication factories differ in structure and some functionalities between viruses, which depend upon the viral genome composition and replication strategy. The components and functionalities of replication factories formed by viruses containing different genomic compositions will be discussed.

1.6.1. Double-stranded RNA viruses

Viruses with dsRNA genomes, such as members of the *Reoviridae* family, induce the formation of cytoplasmic inclusions or factories via the accumulation of specific non-structural proteins. The cytoplasmic factories formed after an infection with orbiviruses, rotavirus and reovirus are named VBs, viroplasms and viral factories (VFs) respectively (Brookes *et al.* 1993; Fabbretti *et al.* 1999; Becker *et al.* 2003). Although they differ in terms of their compositions and interactions, these inclusions all function mainly as sites for viral

genome replication and assembly of progeny virions (Kar *et al.* 2007; Hu *et al.* 2012; de Castro *et al.* 2014), as will be discussed below.

1.6.1.1. *Orbiviruses*

Following an infection with an orbivirus such as AHSV, BTV or epizootic haemorrhagic disease virus (EHDV), cytoplasmic VIBs are formed in infected cells by the non-structural protein NS2. The sequence of Seg-8, which encodes NS2 in orbiviruses, has been determined and compared among BTV, AHSV and EDHV (van Staden *et al.* 1991). Seg-8 of AHSV is 1166 bp in size, and shows 53% homology to BTV and EHDV. NS2 of AHSV is 365 aa, the N-terminal regions being the most conserved, with approximately 58% homology between the different orbiviruses.

Of the ten viral proteins, NS2 is the only phosphorylated protein in AHSV and BTV infected cells (Huisman *et al.* 1987; Devaney *et al.* 1988). For all three orbiviruses, phosphorylation of NS2 has been found to be mediated by a ubiquitous cellular kinase (Theron *et al.* 1994), which in the case of BTV was later identified as casein kinase 2 (CK2), acting specifically on Ser 249 and Ser 259 of NS2 (Modrof *et al.* 2005). In the case of BTV, the phosphorylation and dephosphorylation of NS2 by CK2 and protein phosphatase 2A respectively mediates the dynamic morphology of the VIBs (Modrof *et al.* 2005; Mohl and Roy 2016). NS2 of BTV assemble into large VIBs only when phosphorylated, whereas dephosphorylated NS2 only forms small aggregates (Modrof *et al.* 2005).

Monomeric BTV NS2 are folded into β -sandwich structures, and crystallography has revealed monomer-monomer interactions, enabling the molecules to oligomerise (Butan *et al.* 2004). Although limited literature is available regarding the identification of the multimerisation domains of NS2, the C-terminal of BTV NS2 has been shown to be dispensable for this process (Zhao *et al.* 1994). Electron-dense cytoplasmic aggregates or VIBs are formed by BTV NS2 as early as 4 hours post-infection (h pi), which have been shown to progressively increase in size as infection proceeds, peaking in numerical density at 18 h pi and showing the largest sizes at 24 h pi (Brookes *et al.* 1993). In the case of AHSV and BTV, recombinant expression of NS2 in the absence of other viral proteins is sufficient to form VIB-like structures (Thomas *et al.* 1990; Uitenweerde *et al.* 1995; Kar *et al.* 2007). Although the VIBs in BTV-infected cells have been found to be associated with intermediate filaments (Eaton *et al.* 1987), the VIBs are not dependent on these filaments for proper formation (Kar *et al.* 2007).

BTV NS2 is capable of binding both cellular and viral ssRNA, but not dsRNA (Huisman *et al.* 1987), however BTV NS2 has a higher affinity towards BTV RNAs compared to cellular RNAs (Lymperopoulos *et al.* 2003). A higher degree of phosphorylation of EHDV NS2 corresponds to a decreased affinity for ssRNA (Theron *et al.* 1994), however the phosphorylation status does not affect the ssRNA binding activity of BTV NS2 (Modrof *et al.* 2005). The binding regions of BTV NS2 for ssRNA were identified as residues 2-11 of the amino terminus,

as well as residues 153-166 and 274-286 (Fillmore *et al.* 2002). The affinity of NS2 for RNAs is not dependent on the nucleotide sequence of the RNA (Zhao *et al.* 1994). Rather, the secondary structures of the ssRNA segments account for the binding specificity to NS2 (Lymeropoulos *et al.* 2003). Viral RNAs in secondary structure formation bind to BTV NS2 with a higher affinity compared to unfolded RNAs (Markotter *et al.* 2004). Each segment folds into distinct stem loop structures, which bind at specific regions of NS2 (Lymeropoulos *et al.* 2006). This is suggested to aid in distinguishing between the different genomic segments, to ensure that each of the ten unique viral RNA segments is packaged within the core particles.

The core proteins VP3 and VP7, and the transcriptase complex proteins VP1, VP4 and VP6 colocalise with the VIBs (Modrof *et al.* 2005; Kar *et al.* 2007). This, along with the presence of virus core particles in the matrix of the VIBs (Brookes *et al.* 1993), suggests the role of the VIBs as serving as sites of viral genome replication and core assembly. The core protein VP3 colocalises to VIB-like structures formed by individually expressed NS2, however VP7 only colocalises to the VIB-like structures in the presence of VP3 (Kar *et al.* 2007). VP1 binds as effectively to BTV NS2 in the presence of RNase as in native conditions, indicating that their interactions are independent of RNA binding and rather due to protein-protein interactions (Modrof *et al.* 2005). The phosphorylation status of BTV NS2 does not affect its binding to VP1 (Modrof *et al.* 2005).

The enzymatic activities of NS2 include NTP hydrolysis and nucleotidyl phosphatase activities (Taraporewala *et al.* 2001). The nucleotidyl phosphatase activities might serve to unwind ssRNA stem-loop structures prior to packaging. The cleavage at α , β and γ phosphodiester bonds during nucleoside triphosphate (NTP) hydrolysis is suggested to provide NS2 with energy for the transportation and packaging of ssRNA segments into viral particles within the VIBs. NS2 is indispensable for reverse genetics both in the case of AHSV and BTV, as its absence results in failed virus recovery (van Rijn *et al.* 2016). The VIBs are thus essential for the replication of the orbiviruses.

1.6.1.2. *Rotavirus*

Rotavirus is an enteric virus that mainly infects young children and infants. The rotavirus particle is non-enveloped and triple-layered (Hu *et al.* 2012). The viral genome consists of eleven dsRNA segments, encoding six structural and six non-structural proteins. Following rotavirus infection, both the non-structural proteins NSP2 and NSP5 are essential for the formation of cytoplasmic inclusions termed viroplasms (Fabbretti *et al.* 1999).

NSP2 is a 35 kDa protein which self-assembles into octamers (Butan and Tucker 2010). NSP2 has a sequence-independent affinity for ssRNA, and recruits the viral inner capsid protein VP2, and viral polymerase VP1 (Hu *et al.* 2012). NSP2 is capable of self-phosphorylation (Taraporewala *et al.* 1999), however it can also undergo phosphorylation by cellular casein kinases (Bar-Magen *et al.* 2007). NSP2 possesses NTPase, RNA triphosphatase (RTPase) and helix destabilising activities (Taraporewala *et al.* 1999; Taraporewala and Patton

2001; Vasquez-del Carpio *et al.* 2006) and provides ATP for NSP5 phosphorylation through its nucleoside diphosphate kinase activities (Kumar *et al.* 2007). NSP2 has been shown to depolymerise the microtubule network, leading to tubule granulation, which is supposed to inhibit the trafficking of cellular proteins involved in antiviral defences (Martin *et al.* 2010).

NSP5 exists as different isoforms throughout the rotavirus replication cycle, ranging from a 26 kDa hypophosphorylated form to a 34 kDa hyperphosphorylated form (Afrikanova *et al.* 1996). NSP5 has sequence-independent affinities for both ssRNA and dsRNA (Kattoura *et al.* 1992; Vende *et al.* 2002). NSP5 and viral ssRNAs bind competitively to NSP2 (Jiang *et al.* 2006). In addition, NSP5 associates with the non-structural protein NSP6, which in turn possesses sequence-independent nucleic acid binding affinities.

Effective viroplasm formation requires the hyperphosphorylation of NSP5 (Campagna *et al.* 2007). Hyperphosphorylation of NSP5 can only occur in the presence of NSP2 (Welch *et al.* 1989; Afrikanova *et al.* 1998), and occurs through casein kinase 1 α (CK1 α) (Campagna *et al.* 2007). Dispersedly distributed NSP2 is suggested to interact with hypophosphorylated NSP5, and viroplasm maturation occurs in parallel with phosphorylation of NSP2 and NSP5 (Criglar *et al.* 2014). In addition, CK1 α has been shown to directly affect NSP2, as shown by the knock-down of CK1 α being associated with the disruption of NSP2-NSP5 interactions, restriction of NSP2 to only distribute dispersedly, and an overall decrease in NSP2 protein levels (Criglar *et al.* 2018). Rotavirus viroplasms have been shown to associate with lipid droplets (Cheung *et al.* 2010), which is suggested to be essential for the transition of small foci into large viroplasms (Crawford and Desselberger 2016).

Viral RNAs, inner capsid proteins and RNA transcriptases are recruited to the viroplasms, where viral replication and genome encapsidation occur to form double-layered virions (Hu *et al.* 2012). Thereafter, the newly assembled virions exit the viroplasm and enter the endoplasmic reticulum for maturation and eventual release.

1.6.1.3. *Reovirus*

Reovirus has a broad host range and is frequently used as an experimental model to elucidate the replication and pathogenicity of dsRNA viruses. The reovirus particle is non-enveloped and double-layered (Becker *et al.* 2003). The genome contains ten dsRNA segments encoding eight structural and three non-structural proteins. Following reovirus infection, non-structural proteins σ NS and μ NS, together with minor core protein μ 2, form cytoplasmic inclusions termed viral factories (VFs).

The σ NS protein is 41 kDa in size and binds ssRNA in addition to colocalising with proteins involved in dsRNA synthesis (Becker *et al.* 2001). σ NS binds ssRNA preferentially to double-stranded nucleic acids, however

there seems to be no preferential binding to reovirus RNA over other RNAs (Gillian *et al.* 2000). Through its strand displacement activities, σ NS plays a role in the replication of the viral genome (Gillian *et al.* 2000).

The μ NS protein is 80 kDa in size and has an affinity for ssRNA and viral core particles (Antczak and Joklik 1992; Broering *et al.* 2002). When expressed in the absence of other viral proteins, μ NS is capable of forming large cytoplasmic inclusions (Miller *et al.* 2003). μ NS binds to core particles, allowing viral RNAs within the core particles to undergo transcription and cap acquisition, whilst preventing premature outer capsid assembly (Broering *et al.* 2000).

The core protein μ 2 is encoded by the M1 gene, and in association with other core proteins forms a polymerase complex. μ 2 has also been shown to account for strain-specific transcription temperature optimums (Yin and Cheang 1996). μ 2 binds both ssRNA and dsRNA (Brentano *et al.* 1998) and has NTPase activities (Noble and Nibert 1997).

Reovirus VFs function as sites of viral RNA assortment, genome replication, particle assembly and secondary transcription (de Castro *et al.* 2014). μ NS associates with the minor core protein μ 2, which in turn is associated with the cellular microtubule network (Broering *et al.* 2002). The differential association of μ 2 between two different strains of reovirus, type 1 Lang (T1L) and type 3 Dearing (T3D) with the microtubule network confers distinct morphological characteristics to their VFs, showing filamentous shapes in T1L and more spherical morphologies in T3D infected cells (Parker *et al.* 2002). The association between the VFs and the microtubule network might function to transport essential components to the inclusions, or to transport progeny virions to the plasma membrane for their egress (de Castro *et al.* 2014).

A comparison between the viral factories induced by the different *Reoviridae* members is summarised in Table 1.

Table 1: A comparison between the cytoplasmic inclusions formed by different members of the *Reoviridae* family

	BTB / AHSV	Rotavirus		Reovirus		
Term used to describe inclusion	Viral inclusion body (VIB)	Viroplasm		Viral factory (VF)		
Proteins involved	NS2	NSP2	NSP5	μNS	σNS	μ2
Size (kDa)	41	35	26-35	80	41	83
Phosphorylated	✓ ^[1]	✓ ^[4]	✓ ^[13;14]	?	X	X
Autokinase activity	X ^[2]	✓ ^[4]	✓ ^[7]	?	X ^[9]	X
ssRNA binding activity	✓ ^[1]	✓ ^[4]	✓ ^[5]	✓ ^[18]	✓ ^[9]	✓ ^[11]
NTP hydrolysis activity	Nucleotidyl phosphatase ^[2] at α, β and γ bonds ^[2]	RTPase ^[6] at γ bonds ^[4]	ATPase ^[7]	?	X ^[9]	✓ ^[12]
Association with cytoskeleton	Intermediate filaments ^[3]	Tubulin ^[16]	?	Microtubules (μ2) ^[8]		
Other activities	?	Helix destabilising ^[15] , NDP kinase ^[19]	?	Strand displacement (σNS) ^[9]		
Interaction/ association with organelles	X	Lipid droplets; ER ^[17]		ER; ER-Golgi intermediate, mitochondria ^[10]		

✓ = positive; X = negative; ? = not known.

Refs: [1] Huismans *et al.* 1987; [2] Taraporewala *et al.* 2001; [3] Eaton *et al.* 1987; [4] Taraporewala *et al.* 1999; [5] Kattoura *et al.* 1992; [6] Vasquez-del Carpio *et al.* 2006; [7] Bar-Magen *et al.* 2007; [8] Broering *et al.* 2002; [9] Gillian *et al.* 2000; [10] de Castro *et al.* 2014; [11] Brentano *et al.* 1998; [12] Noble and Nibert 1997; [13] Welch *et al.* 1989; [14] Afrikanova *et al.* 1996; [15] Taraporewala and Patton 2001; [16] Martin *et al.* 2010; [17] Cheung *et al.* 2010; [18] Antczak and Joklik 1992; [19] Kumar *et al.* 2007.

1.6.2. Single-stranded RNA viruses

Positive-sense RNA viruses such as hepatitis C virus, Dengue virus and picornavirus induce the formation of cytoplasmic replication factories by annexing and modifying cell membranes (reviewed by Harak and Lohmann 2015). The membrane alterations involved in replication factory formation mostly entail modification of the endoplasmic reticulum (ER), and are categorised into two main types. The first type is double membrane vesicles (DMV), which are speculated to form from ER exvaginations. The second type is spherule-invagination type vesicles, which are formed from ER invaginations. Hepatitis C virus and picornavirus form DMV-type replication factories, and Dengue fever virus forms spherule-invagination type vesicles. The membranes serve as anchors to the replication factories for proteins involved in viral replication, and shield viral particles against cellular defences.

Negative-sense RNA viruses, like members of the *Mononegavirales* order, form liquid-phase cytoplasmic replication factories with viral nucleoproteins and phosphoproteins. This phase separation prevents cellular antiviral proteins from accessing viral components. Rabies virus forms Negri Bodies, which contain all the components necessary for viral replication, including ribonucleoproteins (Nikolic *et al.* 2017). Human

respiratory syncytial virus forms inclusion bodies (IBs), where viral RNA synthesis occurs. The IBs also compartmentalise mRNA sorting within IB sub-compartments termed inclusion body-associated granules (Rincheval *et al.* 2017). The IBs of human parainfluenza virus are associated with acetylated α -tubulin, which is suggested to modulate the fusion of small foci into larger inclusions (Zhang *et al.* 2017).

1.6.3. DNA viruses

The infection cycle of the majority of DNA viruses is predominantly nuclear-localised. However, the replication of some DNA viruses, like large nucleocytoplasmic DNA viruses, is restricted to the cytoplasm. Viruses of the *Megavirales* order often induce the formation of cytoplasmic viral replication factories via the accumulation of viral DNA enclosed by ER membrane cisternae.

The replication factories of poxvirus are associated with viral proteins involved in DNA replication and transcription, and also provide the sites for viral particle assembly (Schramm and Locker 2005). Mimivirus induces the formation of replication factories as sites for DNA replication, membrane biosynthesis, capsid assembly and genome encapsidation (Mutsafi *et al.* 2013). The Mimivirus replication factories were also found to contain viral proteins involved in transcription and DNA repair (Fridmann-Sirkis *et al.* 2016). The association of the replication factories with the ER is suggested to provide lipids for the formation of the virus capsid. The replication factories are also associated with mitochondria, which could serve as an energy supply for the processes occurring within these factories.

1.7. Eukaryotic translation in a nutshell, and viral translation up-regulation strategies

As viruses do not possess their own translational machinery, they depend upon the cellular translational apparatus to synthesise viral proteins. Viruses compete with the host for protein synthesis machinery, and have adapted various strategies of overthrowing cellular apparatus for their replicative advantage.

1.7.1. An overview of ribosome assembly and translation initiation in eukaryotes

Ribosome assembly in eukaryotes is a complex process and involves multiple steps (reviewed by Peña *et al.* 2017). Within the nucleolus, the RNA polymerase transcribes ribosomal DNA to form 35S pre-rRNA. The 90S pre-ribosome is formed by the association of 35S pre-rRNA with small nucleolar RNAs, the ribonucleoprotein U2 and 40S-specific ribosomal proteins. Co-transcriptional cleavage of the 90S pre-ribosome results in its separation into pre-40S and pre-60S subunits. The pre-ribosomal subunits mature in the nucleus into small 40S and large 60S ribosomal subunits. The ribosome subunits are ultimately exported to the cytoplasm to be utilised for translation.

Eukaryotic translation initiation involves a series of synchronised steps (reviewed by Jackson *et al.* 2010), summarised in Fig. 1.2 . A ternary complex is formed by the association of eIF2 with a transfer RNA (tRNA) associated with methionine (Met) and guanylyl triphosphate (GTP). The small 40S ribosomal subunit associates with the ternary complex, as well as with eukaryotic translation initiation factors eIF1, eIF1A, eIF3 and eIF2, forming the 43S pre-initiation complex (PIC). The initiation factors eIF4A, eIF4E and eIF4G (together forming eIF4F) bind to the 5' cap and unwind the secondary structure of the mRNA. This is followed by binding of the 43S PIC to the 5' cap of the mRNA. The 43S PIC then scans along the 5' untranslated region until reaching the AUG start codon in the Kozak consensus sequence. Once there, GTP is hydrolysed, and the initiation factors dissociate. The large 60S ribosomal subunit attaches to the small 40S subunit to form the 80S ribosome (reviewed by Latchman 2015). The tRNA-Met is at the P site of the ribosome, and the adjacent A site is open to allow the incorporation of a different amino acid, thereby initiating the synthesis of a polypeptide chain (reviewed by Pierce 2014). In eukaryotes, multiple ribosomes can translate the mRNA simultaneously, forming a polyribosome.

Translation initiation is additionally mediated by various cellular factors (reviewed by Latchman 2015). In most cases, the 3' ends of polyadenylated mRNAs link to eIF4G at the 5' cap facilitated by poly (A) binding protein (PABP), facilitating translation re-initiation. Assembly of eIF4E at the 5' cap is mediated by the phosphorylation status of eIF4E-binding protein (eIF4E-BP). When eIF4E-BP is dephosphorylated, it binds to eIF4E and prevents eIF4G and eIF4A from binding to eIF4E and thus hinders the formation of the translation initiation complex. As hydrolysis of GTP following ribosome scanning yields eIF2-GDP, the recycling of eIF2-GTP is required for translation re-initiation. This is facilitated by eIF2B, which acts as guanine nucleotide exchange factor, thereby catalysing the exchange of GDP for GTP. Phosphorylated eIF2 binds tightly to eIF2B, thereby sequestering it and preventing it from exchanging GTP on free eIF2 molecules, leading to the inhibition of translation.

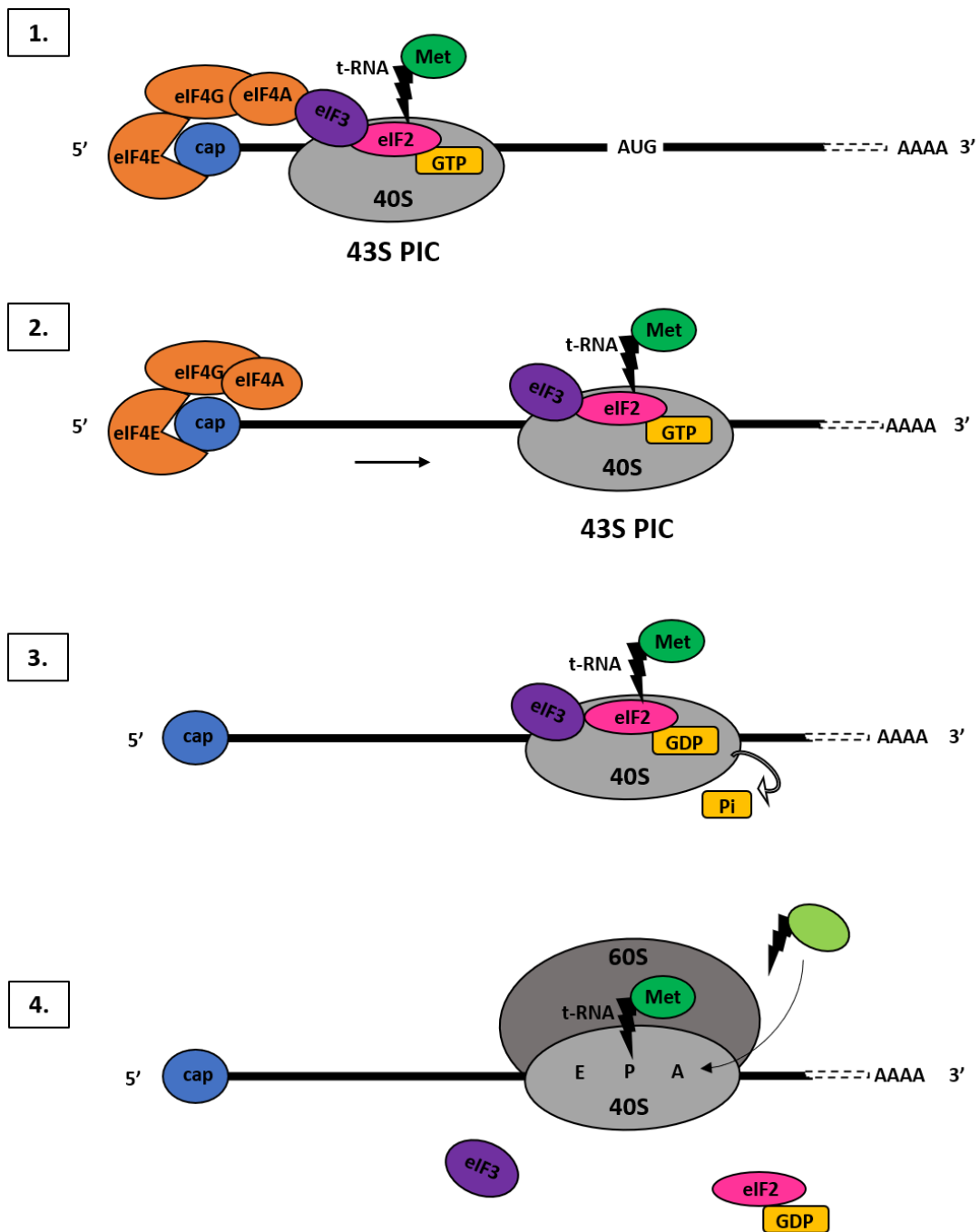


Fig. 1.2. An overview of the process of translation initiation in eukaryotes, adapted from Jackson *et al.* (2010). Step 1: the eukaryotic translation initiation factors (eIFs) eIF4E, eIF4G and eIF4A bind to the 5' cap structure of the mRNA, followed by association of the 43S pre-initiation complex (PIC); step 2: the 43S PIC containing the 40S ribosomal subunit, eIF3 and eIF2 with a tRNA attached to GTP and a methionine (Met) scans along the mRNA until the first AUG start codon is reached; step 3: GTP is hydrolysed, and initiation factors dissociate from the 40S ribosome; step 4: the 60S subunit joins and a new tRNA associated with a second amino acid is incorporated at the A site. For simplicity, not all eukaryotic translation factors were included.

1.7.2. Viral strategies of subverting the host defence mechanisms and promoting viral translation

Upon cell stress, such as that induced by a viral infection, cellular defence mechanisms are activated to counter the stress stimulus. A common defence mechanism is the overall suppression of protein synthesis by the host. Viruses have, however, adapted strategies to overcome these defences in various ways to ensure effective replication.

Various viruses produce RNAs that resemble eukaryotic mRNAs, which serve to recruit the host translation components to translate the viral RNAs. Some viruses like members of the *Reoviridae* family produce their own 5' caps via virus-encoded guanylyl methyltransferases (Furuichi *et al.* 1975; Ramadevi *et al.* 1998). Other viruses transfer 5' caps from eukaryotic mRNAs to viral RNAs, or synthesise small viral-encoded proteins to function as 5' cap substitutes (Walsh *et al.* 2013). In addition to 5' caps, some viruses produce RNAs that are polyadenylated (Walsh *et al.* 2013). Rotavirus non-structural protein NSP3 mimics PABP to some extent and links the 3' end of viral RNA with eIF4G, thereby promoting viral translation re-initiation (Walsh *et al.* 2013).

Some viruses suppress host translation by inhibiting the formation of the 5' cap-initiation complex (Schneider and Mohr 2003). Picornavirus encodes proteases that cleave eIF4E, thereby preventing it from binding to eIF4G and PABP. While this cleavage leads to the inhibition of cellular translation, viral translation is not inhibited, as the cleaved terminal of eIF4G binds with a high affinity to picornavirus RNA. Dengue fever virus, reovirus and picornavirus suppress host translation by inducing the dephosphorylation of eIF4E-BP, in effect preventing essential components from assembling at the 5' cap initiation complex (Roberts *et al.* 2009). Some viruses prevent the linkage of the polyadenylated 3' end of eukaryotic mRNA to the 5' caps. Picornavirus and calicivirus encode proteases that cleave PABP, and in cells infected with herpesvirus or rotavirus, PABP accumulates in the nuclei. The removal of PABP in effect inhibits host translation re-initiation.

Many viruses, including cricket paralysis virus and human immunodeficiency virus, are capable of initiating translation independent of the cap-binding complex. This is done by initiating translation at an internal ribosome entry site (IRES) situated at a distance away from the 5' terminus of the viral RNA (Schneider and Mohr 2003). Infectious bursal disease virus is capable of initiating translation independent of a 5' cap through its viral structural proteins VP1 and VP3 (Ye *et al.* 2018). These proteins mediate the recruitment of host factors and allow access of ribosomal components towards an internal start codon.

Viruses also interfere with host translation by altering the phosphorylation state of translation factors. In avian reovirus-infected cells, eIF2 is phosphorylated extensively whereas eIF4E, eIF4B and 4E-BP are dephosphorylated, resulting in the inhibition of host translation (Ji *et al.* 2009). Often when the host cell detects a viral infection, it stimulates the expression of RNA-regulated protein kinases (PKR) (Schneider and Mohr 2003). PKR mediates the phosphorylation of eIF2, preventing the recycling of eIF2-GTP, and subsequently inhibiting both host and virus translation re-initiation. Adenovirus encodes a small nucleotide

that competitively binds to the RNA-binding domain of PKR, thereby inhibiting its activity. Herpes simplex virus, reovirus, poxvirus and influenza virus encode RNA-binding complexes, which mask viral dsRNAs from PKR phosphorylation (Schneider and Mohr 2003). In addition, reovirus non-structural protein σ NS sequesters dsRNA and prevents the activation of PKR (Schmechel *et al.* 1997).

Another cellular defence mechanism is the formation of stress granules (SGs), which store cellular components such as mRNAs, ribosomes and translation factors in an inactive state to limit their damage (Reineke and Lloyd 2013). However, some viruses are capable of circumventing this response or using it to their advantage. The induction of SG formation during early stages of reovirus infection (Buchan and Parker 2009) has been shown to be mediated by the phosphorylation state of eIF2 α (Qin *et al.* 2009). During late stages of reovirus infection, SGs have been shown to be disrupted, which is independent of eIF2 α phosphorylation (Qin *et al.* 2011). Reovirus VF protein μ NS associates with the SGs (Carroll *et al.* 2014), and σ NS associates with the G3BP protein involved in SG formation (Choudhury *et al.* 2017). In addition, rotavirus viroplasms have been shown to be associated with many SG-associated proteins (Dhillon *et al.* 2018). The disruption of SGs and/or active sequestration of essential translational components from SGs in effect promotes viral translation.

A range of different viruses compartmentalise translation within their cytoplasmic replication factories. In this way, viruses sequester host translation components away from host mRNAs towards regions where viral transcripts are abundant. This results in promoting virus protein synthesis while inhibiting host translation.

In reovirus-infected cells, viral translation was shown to be compartmentalised within their VFs (Desmet *et al.* 2014). Various translation initiation, elongation and termination factors were found to colocalise with μ NS. Translation initiation factor eIF4E and ribosomal proteins L11 and rpS3 were shown to be distributed throughout the viral factories, whereas eIF3A and the ribosomal proteins RiboP and pS6R were localised to the margins of the VFs. It was found that eIF3A and pS6R colocalised with σ NS, and that they co-immunoprecipitated, indicating protein-protein interactions. Active viral protein synthesis was shown to occur within the VFs, as seen by the strong colocalisation of ribopuromycylated polypeptides with the VFs. Based on this, a model was proposed in which the viral mRNAs associate with eIF4E within the factories, and then become associated with the 43S PIC at the margins, followed by the addition of the ribosomal components, which permeate through the viral factory, initiating translation.

Poxviruses VFs have been shown to be associated with eIF4E and eIF4G and to be the sites of viral translation (Katsafanas and Moss 2007). African swine fever virus (ASFV) was shown to sequester the translation initiation factors eIF4E, eIF4G, eIF2, eIF3b and elongation factor eEF2, as well as ribosomal components to their viral factories. Additionally, mitochondria were shown to localise to the ASFV viral factories, providing ATP in proximity for viral replication and translation (Sánchez *et al.* 2013). Cauliflower mosaic virus synthesises viral proteins within inclusion bodies. The inclusion bodies of Cauliflower mosaic virus are

surrounded by ribosomes, and the protein forming the inclusions was shown to bind to various proteins constituting the 60S ribosomal subunit and to various translation factors (Schoelz and Leisner 2017).

Much is yet to be determined about how orbiviruses up-regulate virus protein synthesis. One of the earliest molecular studies done on BTV already indicated that BTV inhibits host protein synthesis (Huismans 1971). This was shown to result from degrading polyribosomes, either by directly breaking down complexes or preventing the assembly of new polyribosomes. Pulse-labelling BTV-infected cells with ^{14}C revealed that from 11 h pi, more viral proteins than cellular proteins are synthesised (Huismans 1979). In the case of AHSV, pulse-labelling with ^{35}S -methionine revealed that the bulk of protein synthesis becomes predominantly virus-specific from 13 h pi onwards (van Staden *et al.* 1995). NS1 enhances bluetongue virus translation, with the mechanisms still to be elucidated (Boyce *et al.* 2012).

1.8. Concluding remarks

The orbivirus African horse sickness virus (AHSV) causes serious disease in horses. It remains to be understood how AHSV up-regulates virus protein synthesis following infection to promote the viral replication cycle. AHSV induces cytoplasmic replication factories named viral inclusion bodies (VIBs) via accumulation of the non-structural protein NS2. The time-frame within which these VIBs form, and how their numbers and/or size change during the course of infection, have not yet been described for AHSV. The VIBs function as sites for the replication of the viral genome and the packaging of virus core particles to produce progeny virions. Thus far, no other functions have been reported for orbivirus VIBs.

The formation of cytoplasmic viral replication factories is a property exhibited by a range of different viruses. These all share similar core functionalities, and are aligned with those described for the VIBs. However, various different viral replication factories have been shown to also sequester components of the host translation apparatus and to become the sites of virus protein synthesis. This compartmentalisation of virus translation in effect serves to promote the viral replication cycle whilst suppressing host protein synthesis.

As the VIBs are essential for AHSV replication, it is important to understand how and when they are formed. It remains to be explored whether AHSV VIBs have additional functions as sectioning and promoting virus protein synthesis. Exploring this avenue could elucidate an additional mechanism whereby AHSV enhances the viral infection cycle.

1.9. Aims and objectives

The aim of this study was to further our understanding of the contribution of AHSV VIBs to the viral replication cycle, with a specific focus on investigating whether AHSV VIBs interact with the cellular translation apparatus to become sites of virus protein synthesis. To address this aim, the following objectives were outlined for this study:

- 1.** To characterise the distribution, morphology and abundance of AHSV VIBs as infection proceeds, and investigate how the VIBs are formed.
- 2.** To investigate whether the VIBs of AHSV are the sites of active protein synthesis.
- 3.** To investigate whether specific components of the host translation apparatus are found within the VIBs.
- 4.** To investigate whether the interaction between the VIBs and the host translation apparatus is mediated exclusively by NS2.

Chapter 2

2.1. Introduction

Following AHSV or BTV infection, large dense cytoplasmic matrices known as the viral inclusion bodies (VIBs) are formed by non-structural protein NS2 (Brookes *et al.* 1993; Uitenweerde *et al.* 1995). The exact mode of how VIBs are assembled from NS2 precursors is not yet understood. Thus far, the only known function of the orbivirus VIBs is to serve as sites for viral genome replication and assembly of genomic segments into core particles (Kar *et al.* 2007). Cytoplasmic structures functionally similar to the VIBs are formed during infections with other members of the *Reoviridae* family (Butan and Tucker 2010; de Castro *et al.* 2014). In the case of reovirus, the replication factories are termed viral factories (VFs) and are formed by the interaction of viral non-structural proteins σ NS and μ NS (Fabbretti *et al.* 1999). In the case of rotavirus, replication factories named viroplasms are formed by the interaction of non-structural proteins NSP2 and NSP5 (Becker *et al.* 2003).

After entering the cell and disassembling the outer capsid proteins, orbivirus core particles become transcriptionally active, and produce positive-sense ssRNAs (Patel and Roy 2014) representing each of the 10 genome segments. The ssRNAs are capped and extruded co-transcriptionally from the core particles into the cytoplasm, where they either interact with cellular apparatus to be translated into viral proteins, or are recruited to the VIBs in distinct secondary structure conformations (Huismans *et al.* 1987; Ramadevi *et al.* 1998; Lympieropoulos *et al.* 2006). Along with the ssRNAs, core proteins VP3 and VP7 and the transcriptase complex proteins VP1, VP4 and VP6 are also recruited to the VIBs (Kar *et al.* 2007). Within the VIB matrix, the full complement of ssRNAs along with the transcriptase complex proteins are packaged into core particles, where the ssRNAs are replicated to produce the dsRNA genomic segments. These assembled cores are transcriptionally active, resulting in newly synthesised transcripts localised within the VIBs (Kar *et al.* 2007). This second wave of transcription after the primary infection allows for exponential viral growth. Once the assembled cores are released from the VIBs into the cytoplasm, they acquire outer capsid proteins VP2 and VP5. This abolishes further transcription, and the mature viruses egress from the cell.

Successful viral replication requires efficient take-over of host translational machinery to favour virus protein synthesis. The prototype virus, BTV, was shown to inhibit host protein synthesis soon after infection by degrading polyribosomes (Huismans 1971). Translation was shown to become predominantly virus-specific from 11 h pi onwards in the case of BTV (Huismans 1979) and from 13 h pi onwards in the case of AHSV (van Staden *et al.* 1995). NS1 has been shown to be a positive regulator of BTV translation, either by enhancing the stability of BTV RNA or by increasing the rate by which viral RNAs are used as templates for translation (Boyce *et al.* 2012; Kerviel *et al.* 2019). Much more is yet to be elucidated about how orbiviruses accomplish the take-over of host translation.

The replication factories of both rotavirus and reovirus were shown to interact with specific cellular proteins involved in either the execution or regulation of protein synthesis. Reovirus VFs (Carroll *et al.* 2014; Choudhury *et al.* 2017) and rotavirus viroplasms (Dhillon and Rao 2018) interact with proteins that induce the formation of stress granules, suggesting the annexation of essential cellular components from their inactive fates towards promoting viral replication. Moreover, reovirus VFs were shown to sequester ribosomal components as well as host initiation, elongation and termination factors and to thereby become the sites of virus protein synthesis (Desmet *et al.* 2014). The compartmentalisation of viral translation within cytoplasmic viral replication factories has also been reported for poxvirus, African swine fever virus and Cauliflower mosaic virus (Katsafanas and Moss 2007; Sánchez *et al.* 2013; Schoelz and Leisner 2017). This strategy in essence allows for essential cellular machinery to be hijacked for preferential enhancement of virus protein synthesis.

The main focus of this study was to investigate whether the VIBs of AHSV can be involved in up-regulating virus protein synthesis. It was first examined how the VIBs form and mature during the advancement of the AHSV infection cycle. This was taken further by characterising how large VIBs are formed from small punctate NS2 foci in live cells. It was then assessed whether actively translating ribosomes are found within the VIBs, and investigated whether a number of cellular translation components colocalise with NS2 to the VIBs or compartments of the VIBs. The results obtained could introduce a novel function of the VIBs, and elucidate a possible mechanism whereby AHSV enhances its replication cycle.

2.2. Materials and Methods

2.2.1. Cells

BSR-T7 mammalian cells (a clone of BHK-21 cells (ATCC CCL-10), expressing a T7 polymerase (Buchholz *et al.* 1999) were obtained from Dr AC Potgieter (Deltamune [Pty] Ltd) and cultured in Minimal Essential Medium (MEM, HyClone) supplemented with 5% foetal calf serum, 1.2% fungizone, 1% penicillin-streptomycin and 1% non-essential amino acids. Geneticin was added approximately every 10th division to maintain the T7 polymerase. Cells were cultured as a monolayer on a tissue culture dish, maintained at 37°C in a humidified incubator with 5% CO₂.

Spodoptera frugiperda (Sf9) insect cells (ATCC CRL-1711) were maintained in suspension as spinners cultures in TC100 medium (Gibco) supplemented with 10% foetal calf serum, 1.2% fungizone, 1% penicillin-streptomycin and 1% non-essential amino acids at 28°C.

2.2.2. Virus stock maintenance and titre determination

African horse sickness virus serotype 4 (AHSV-4 OIE reference strain, isolate 32/62) and serotype 5 (AHSV-5 OIE reference strain, isolate 30/62) and bluetongue virus serotype 10 (BTV-10) were amplified by inoculating confluent BSR-T7 monolayers in a T75 flask with virus, and harvesting after complete cytopathic effect (CPE) had occurred. Cells were passed through a 22G needle to lyse the cells and release the intracellular virus particles, and viral stocks were stored at 4°C. Wildtype baculovirus (BacWT) and a recombinant baculovirus expressing AHSV-9 NS2 (BacNS2) (Uitenweerde et al, 1995) stocks were amplified by inoculating confluent Sf9 cells in a T75 flask with virus, and harvesting after complete CPE had occurred. Viral stocks were stored at 4°C.

The viral titre was determined via 50% tissue culture infectious dose (TCID₅₀) endpoint dilution assay. Briefly, it involved adding serially diluted virus stock (10^{-1} to 10^{-7}) in increasing dilutions to rows across plates containing cell monolayers. The inoculums were incubated for 6-8 days and scored for the presence of CPE with a phase contrast inverted microscope. The titre was calculated with the Reed-Muench method (Reed and Muench 1928).

2.2.3. Plasmid construction for transient expression system

A plasmid containing NS2-eGFP downstream of a CMV promoter was constructed for transient expression of the NS2-eGFP fusion protein. The backbone plasmid phCMV-NS2 (GenScript Corporation) was kindly provided by Dr AC Potgieter (Deltamune [Pty] Ltd). Plasmid phCMV-NS2 contained an AHSV NS2 open reading frame (ORF) codon-optimised for expression in hamster cells cloned into the phCMV-Dream vector under control of a CMV promoter. A phCMV-NS4-eGFP plasmid (kindly provided by Ms S Boughan, University of Pretoria) was used as the source for the eGFP ORF to be inserted. The eGFP ORF was preceded by an upstream serine-glycine (S-G) linker. Primers were designed (purchased from Whitesci) to selectively amplify and generate linearised vector and insert sequences with homologous 5' overhangs, details of the primers are provided in Table 2. Using the specific primers, the phCMV-NS2 and phCMV-NS4-eGFP plasmids were PCR amplified and linearised using Phusion High Fidelity DNA Polymerase (Thermo Scientific), using PCR reaction conditions and cycling parameters according to the manufacturers' instructions. The PCR products were subjected to PCR clean-up using the Zymo DNA Clean & Concentrator Kit (Zymo Research), a column-based DNA purification kit, as specified by the manufacturers. Using In-Fusion® HD Cloning Kit (Takara Bio), the eGFP insert fragment was fused downstream of the NS2 ORF of the phCMV-NS2 vector sequence according to the manufacturers' instructions. In brief, this entailed setting up a cloning reaction containing In-Fusion Enzyme Premix with the linearised vector and insert fragments (2:1), and incubating at 50°C for 15 min in a thermal cycler. Fusion of the insert and vector sequences in principle occurs through annealing of complementary ends.

Table 2. Descriptions of the primers used to amplify vector and insert sequences in preparation for fusion

Primer name	Sequence (5' -> 3')	Description	T _m (°C)
phCMVNS2_vf	TAGTAAATAAAGCAATAGCAT CACAAATTTAC	Forward primer, anneals to the vector sequence downstream of the stop codon of the AHSV NS2 ORF	55.2
phCMVNS2_vr	GCCGGAGCCGCCGGC	Reverse primer, anneals to the vector sequence directly upstream of the stop codon of the AHSV NS2 ORF	67.7
SG_eGFP_if	GCCGGCGGCTCCGGCGGAGGA GGAGGTTCTGGAG	Forward primer, the 3' end anneals to the S-G linker and the 5' end contains sequence homology to the vector sequence	75.7
SG_eGFP_ir	TTGCTTTATTTACTATCA TTTATACAGTTCATCCATCC	Reverse primer, the 3' end anneals to the end of the eGFP ORF including the stop codon, and the 5' end contains sequence homology to the vector sequence	57.2

T_m= primer melting temperature; vf= vector forward; vr= vector reverse; if= insert forward; ir= insert reverse.

2.2.4. Transformation and plasmid isolation

The phCMV-NS2 plasmids were transformed into StrataClone™ competent cells (Agilent), phCMV-NS4-eGFP and phCMV-NS2-eGFP plasmids were transformed into Stellar™ competent cells (Clontech). Plasmid DNA was mixed with competent cells and incubated on ice for 20-30 min, then subjected to heat shock for 45 seconds at 42°C, and returned to ice for 2 min. The transformation reactions were diluted in pre-warmed LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) or SOC medium (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 20 mM MgSO₄), and incubated with agitation (160-225 rpm) for 1 hour at 37°C. The cultures were plated on LB-agar plates supplemented with 100 µg/ml ampicillin, and incubated overnight (O/N) at 37°C. Individual resistant colonies were selected from the plates, and grown in LB medium supplemented with 100 µg/ml ampicillin at 37°C with agitation O/N. The transformed cell suspensions were either used directly or stored at in 50% glycerol at -80°C for longer term storage.

Plasmids were extracted using HighPure Plasmid Isolation Kit (Roche), or GeneJet Plasmid Midi Prep Kit (Thermo Fisher Scientific) for larger volumes of plasmids, according to the protocols provided by the manufacturers. This entailed obtaining bacterial cell pellets via centrifugation, followed by cell lysis with provided buffers. The lysates were passed through columns and subjected to multiple wash steps with centrifugation. DNA was ultimately eluted from columns with ddH₂O.

2.2.5. Restriction enzyme digestion

The phCMV-NS2 plasmids were subjected to restriction enzyme digestion with HindIII restriction endonuclease (Roche), and phCMV-NS2-eGFP plasmids were digested with EcoRI FastDigest (Thermo Scientific), according to the manufacturers' instructions.

2.2.6. Agarose gel electrophoresis

DNA samples were mixed with 6 x DNA Loading Dye (Thermo Scientific) and subjected to 1% agarose gel electrophoresis with 0.5 µg/ml EtBr and 1 x TAE (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA) at 90 V until adequate separation of DNA was obtained. Quick-Load® DNA Ladder (N0469S, New England Biolabs) was used as molecular weight reference.

2.2.7. Transfections

BSR-T7 cells were seeded on coverslips in 24-well plates, 6-well or 30 mm diameter plates. Transfections were conducted when the cells were 70-80 % confluent. For cells on coverslips, MEM was replaced with 1 ml Opti-Mem® Reduced Serum Medium (Gibco), and incubated for 1 hour at 37°C. Round bottom tubes were used for transfections. To two separate tubes containing 30 µl Opti-Mem®, 0.6 µg DNA (phCMV-NS2 or phCMV-NS2-eGFP) was added to the one tube and 1,5 µl Lipofectamine® 3000 (Invitrogen) was added to the other. Both mixtures were mixed separately by pipetting, and incubated at room temperature (RT) for 5 min. The DNA and lipid mixtures were added together and mixed by tilting and incubated for 20 min at RT. Opti-Mem® on the cells was replaced with 0,2 ml Opti-Mem® to just cover the monolayers. The entire volumes of DNA-lipid complexes were added drop-wise to the cells. After 6-24 hours, Opti-Mem® was replaced with MEM and cells were maintained at 37°C with 5% CO₂ and humidity until use. For cells in 6-well or 30 mm diameter plates, the same procedure was followed using 5 x the volumes as indicated.

2.2.8. Ribopuromycylation assay

The ribopuromycylation (RPM) assay was conducted as previously described (David *et al.* 2012; Desmet *et al.* 2014). Briefly, medium was removed from infected or transfected cell monolayers, and cells rinsed once with MEM. Cells were then incubated with pre-warmed MEM supplemented with 208 µM emetine dihydrochloride hydrate (Sigma-Aldrich) for 15 min at 37°C. This was followed by incubation with MEM supplemented with 208 µM emetine and 182 µM puromycin dihydrochloride (Sigma-Aldrich) for 5 min at 37°C. Immediately thereafter, cells were placed on ice and rinsed with chilled 1 x PBS (phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄·2H₂O, 1.8 mM KH₂PO₄, pH 7.4). Cells were simultaneously permeabilised and fixed with chilled permeabilisation buffer (50 mM Tris-HCl [pH 7.5], 25 mM KCl, 5 mM MgCl₂, 355 µM cycloheximide, 5 U/µl Pepstatin, 10 U/ml RNase Out (Thermo Scientific)

ribonuclease inhibitor and 0.2% Triton X-100), supplemented with 4% (w/v) PFA, for 20 min at 4°C. This was followed by immunofluorescence processing.

2.2.9. Antibodies

Immunolabelling was conducted with different antibodies. The details of the antibodies used for specific applications are indicated in Table 3.

Table 3: Antibodies used in different procedures

Primary antibody	Origin	Reference/ Manufacturer	Concentration used		Secondary antibody used	
			IF	IB	IF	IB
α -AHSV NS2	Rabbit polyclonal	Uitenweerde <i>et al.</i> (1995)	1:500	1:500	AlexaFluor 488 or AlexaFluor 594 goat-anti-rabbit (1:250)	
α -BTV NS2		Uitenweerde <i>et al.</i> (1995)			AlexaFluor 488 goat-anti-rabbit (1:250)	
α -PMY (4G11)	Mouse monoclonal	Merck MABE342	1:100	N/A	AlexaFluor 594 goat-anti-mouse (1:250)	Polyclonal rabbit anti-mouse Immunoglobulin HRP (Dako) 1:1000
α -L11 (2A1)	Mouse monoclonal	Santa Cruz sc-293224	1:100	N/A		
α -S3 (E-6)	Mouse monoclonal	Santa Cruz sc-376098	1:250	1:100		
α -eIF4E (A-10)	Mouse monoclonal	Santa Cruz sc-271480	1:250	1:100		
α -eIF3 θ (E-1)	Mouse monoclonal	Santa Cruz sc-365789	1:100	1:50		

α = anti; PMY= puromycin; IF= Immunofluorescence; IB= Immunoblotting

2.2.10. Immunofluorescence

Cells were cultured on glass coverslips in 24-well plates, and when 80-90% confluent, infected or transfected with the appropriate virus (MOI 0,5) or plasmid depending on the application. BSR-T7 cells, except those subjected to the RPM assay, were fixed with 4% PFA for 30 min at room temperature (RT). This was followed by permeabilisation with 0.2% Triton X-100 for 10 min at RT. Sf9 cells were either fixed with 4% PFA and permeabilised with 0.2% Triton X-100 using the same procedure as described above, or subjected to fixation and permeabilisation with cold 1:1 methanol-acetone for 2 min at RT.

To prevent non-specific binding of the antibodies, samples were incubated in a blocking solution consisting of 5% (w/v) milk powder (MP) in 1 x PBS for 30 min at RT. For immunolabelling, the coverslips were transferred to a humidified container. To label a single antigen, the appropriate primary antibody diluted (1:100-1:500) in 1% MP was added and incubated at least 3 hours to O/N at 4°C. The cells were rinsed three

times with wash buffer (0.05% Tween-20 in 1 x PBS). This was followed by incubation with the appropriate secondary antibody diluted (1:250) in 1% MP for 1 hour at RT. Cells were rinsed twice with wash buffer and twice with 1 x PBS. For labelling a second antigen in the same cell, the procedure was repeated using a second set of appropriate primary and secondary antibodies, including blocking and all the rinsing steps. Nuclei were stained with DAPI (10 µg/ml) in 1% MP for 10 min at RT. Cells were rinsed with 1 x PBS, mounted onto microscope slides with Vectashield mounting medium (Vector Laboratories) and sealed with clear adhesive.

2.2.11. Confocal laser scanning microscopy

Samples were analysed with a Zeiss™ LSM 880 confocal laser scanning microscope coupled with an Airyscan detector. AlexaFluor 488 was excited with a 488 nm laser, AlexaFluor 594 with either a 561 nm or 633 nm laser, and DAPI with a 405 nm laser. Plan-Apochromat 40x/NA 1.3 or 100x/NA 1.46 oil objectives were used.

When images were acquired at the different optical planes (through acquisition of a Z-stack), they were merged into a single image by creating a Maximum Intensity Projection (MIP) using Zen Black™ 2.3 software. From the MIPs, the VIBs, represented by green foci, were counted and the surface area of each was calculated automatically with the Zen Blue™ 2.3 software's Image Analysis module. Background reduction was done by excluding foci with surface areas less than 100 nm².

To obtain intensity plot profiles, a path of interest was selected on the micrograph in Zen Blue™ 2.3 software's Profile module, and intensities of fluorophores across the paths were presented on the y-axes.

2.2.12. Quantifying VIB sizes and numerical densities

Microsoft Excel was used to calculate the average number of VIBs per cell and to assign size categories to the VIBs based on surface areas. VIBs with a surface area of at least 100 nm² and smaller than 0,5 µm² were categorised as *small*, equal to 0,5 µm² and smaller than 1 µm² *medium* and equal to or larger than 1 µm² *large*.

2.2.13. Live-cell imaging

BSR-T7 cells were seeded in 30 mm diameter plates with a 0.18 mm thickness glass base. When confluent, cells were transfected with phCMV-NS2-eGFP. From 6 hours post transfection (h pt) onwards, cell plates were placed in a Zeiss™ LSM 880 microscope, with the optimal conditions to maintain mammalian cell growth (37°C, 5% CO₂ and humidity) controlled by the PCON growth chamber and accessories. The 488 nm laser was used to detect NS2-eGFP foci. Definite focus was applied to limit focus drift by the objective. Images were recorded of the live cells at different areas of the plate in intervals of 2 min over a period of 72 hours.

2.2.14. Preparing cell lysates

Confluent cell monolayers were infected or transfected and harvested at specific post-infection or post-transfection time points, depending on the application. Different cell lysing buffers were used and procedures done depending on the experiment.

Cell pellets were collected by centrifugation (Sigma 1-14 K refrigerated centrifuge, rotor 12094) at 4000 rpm, 4°C for 10 min. The pellets were rinsed twice by resuspension in 1 x PBS followed by centrifugation at 3000 rpm at 4°C for 5 min. For immunoblotting, cells were resuspended in hypotonic lysis buffer (0.5% Triton-X, 10 mM Tris-HCl pH 7.4, 1 mM EDTA pH 8, 150 mM NaCl). For immunoprecipitation, cells were resuspended in immunoprecipitation (IP) buffer (150 mM Tris-HCl, 50 mM NaCl, 5 mM MgCl₂ and 1% Triton-X, supplemented with 10 U/ml RNase inhibitor, 1 x Halt Proteinase and Phosphatase inhibitor (Thermo Scientific) and 25 U/ml Benzonase nuclease (Merck)). The cell suspensions were sheared with a 22G needle attached to a syringe, and incubated with agitation at 4°C for 30 min. The lysates were either used directly or stored at -20°C for later use.

For cell lysates obtained directly with 6 x protein solvent buffer (PSB) (0.188 M Tris pH 6.8, 6% SDS, 30% Glycerol, 15% 2-Mercaptoethanol), cell monolayers were resuspended in 6 x PSB, passed through a 22G needle attached to a syringe, and incubated with agitation at 4°C for 30 min. Cell debris were pelleted by centrifugation at 4000 rpm at 4°C for 10 min, and the supernatant was used directly.

2.2.15. Cross-linking

Cell pellets of AHSV-5- infected BSR-T7 monolayers were collected by centrifugation at 4000 rpm at 4°C for 10 min. The pellets were rinsed twice by resuspension in 1 x PBS followed by centrifugation at 3000 rpm at 4°C for 5 min. Cell pellets were incubated with 0.8% PFA dissolved in PBS with moderate agitation for 7 min at RT and centrifuged at 1800 rcf for 3 min at RT. The pellets were quenched with 125 mM glycine dissolved in PBS and centrifuged at 3000 rpm for 5 min. The pellets were lysed with IP buffer, mechanically sheared with a 22G needle and agitated for 30 min at RT before being subjected to SDS-PAGE.

2.2.16. Immunoprecipitation

NS2 was purified from AHSV-5 infected samples using protein-A magnetic beads (S1425S, New England Biolabs). To pre-clear cell lysates from potential non-specific binding of proteins to the beads, 200 µl crude cell extract was added to 25 µl beads, the suspension vortexed and incubated at 4°C for 1 hour. A magnetic field was applied for 30 seconds, and the supernatant was transferred to a clean 1.5 ml microcentrifuge tube. A volume of 25 µl of α-NS2 serum was added to the lysate, the solution was vortexed and incubated at 4°C for 1 hour. A volume of 25 µl fresh magnetic beads was added to the suspension, vortexed and incubated at 4°C with agitation. A magnetic field was applied, the supernatant removed and the bead pellet washed twice

with chilled IP buffer. The beads were resuspended in 6 x PSB and incubated at 70°C for 5 min to denature the proteins bound to the beads. The immunoprecipitated complexes were subjected to SDS-PAGE.

2.2.17. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Cell lysates were resuspended in 6 x PSB and incubated at 90°C for 10 min in a thermal cycler. The samples were loaded into a denaturing gel consisting of a 10% to 12% separating gel phase (10% or 12% of 30% acrylamide-bis ready-to-use solution [37.5:1, Merck], 1.5 M Tris pH 8.8, 0.4% SDS), and a 5% stacking gel phase (5% of 30% acrylamide-bis ready-to-use solution [37.5:1, Merck], 0.5 M Tris pH 6.8, 0.4% SDS), each phase was solidified by the addition of 0.008% (v/v) N,N,N',N' tetramethylethylene diamine and 0.08% (m/v) ammonium persulphate. The gel was submerged in 1 x TGS, (2.5 mM Tris, 19.2 mM glycine, 1% SDS) and gel electrophoresis was performed at 130 V and 400 mA until for 1-2 hours or until desired separation of proteins were obtained. A PageRuler™ Prestained Protein Ladder, (#SM0671; Fermentas) was used as a reference marker.

2.2.18. Immunoblotting

Following SDS-PAGE separation, proteins were transferred from the gel to a Hybond™ C extra nitrocellulose membrane (Amersham Biosciences) at 100 V and 400 mA for 1 hour in Townsin's transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). Blocking was performed with 1% MP in 1 x PBS for 30 min with agitation to prevent non-specific binding of the antibody. The blocking solution was removed, and the membrane incubated in the appropriate primary antibody diluted (1:50-1:500) in 1% MP O/N with agitation. The membrane was washed with three changes of wash buffer for 5 min each. The membrane was incubated with Protein A horseradish peroxidase conjugate (Calbiochem) diluted 1:10 000 in 1% MP in the case of blotting for NS2, or the appropriate secondary antibody diluted (1:1000-1:10 000) in 1% MP when blotting for the other antigens, for 1 hour with agitation. The membrane was rinsed three times with wash buffer for 5 min each wash, followed by one wash with 1 x PBS for 5 min. The membrane was incubated in an enzyme-substrate solution (60 mg 4-chloro-1-naphtol dissolved in 20 ml ice-cold methanol and 60 µl hydrogen peroxide dissolved in 100 ml 1 x PBS) and kept in the dark until the bands became visible. The membrane image was recorded Gel Doc™ Molecular Imager (BioRad).

2.2.19. Quantifying band intensities from immunoblots

To quantify the intensity levels of NS2 and actin, Gel Doc™ images of the immunoblot membranes were analysed with ImageLab, which automatically determined the relative intensities of selected bands.

2.3. Results

The aim of this study was to investigate whether the VIBs of AHSV are sites for virus protein synthesis. Firstly, the VIBs of AHSV were characterised based on their morphology and distribution as infection progressed. Next, the distribution profile of actively translating ribosomes were characterised relative to the VIBs. It was then assessed whether specific components of the host protein synthesis machinery localised to the VIBs or sub-structural domains of the VIBs. Throughout the study, the characteristics of the VIBs formed within the context of an AHSV infection were compared to VIB-like structures resulting from recombinant expression of NS2 in the absence of other AHSV proteins and nucleic acids.

2.3.1. The formation of AHSV virus inclusion bodies is a time-dependent and dynamic process

In cells infected with BTV, the prototype virus for the *Orbivirus* genus, the sizes and quantities of the VIBs as infection proceeded over time had previously been described (Brookes *et al.* 1993). In contrast, the progression of VIB formation over the course of an AHSV infection cycle has not yet been characterised. In addition, the exact mode of how AHSV NS2 assembles to form the large cytoplasmic matrices known as the VIB is not yet understood. These questions were addressed in this section.

2.3.1.1. *NS2 punctate foci aggregate as infection progresses to ultimately form large VIBs*

Initial experiments focussed on the expression of NS2 and the specificity of the available antibodies towards its detection. BSR-T7 cells were infected with AHSV-4, AHSV-5 or BTV-10, and lysates were subjected to Western blotting with anti (α) -AHSV NS2 or anti-BTV NS2 serum respectively. In all cases a specific band of the expected size, 42 kDa, was observed (Fig. 2.1). Faint background bands, single in the case of AHSV and multiple in the case of BTV-infected cells, were detected and is suspected to be the result of protein degradation during sample preparation. It could be concluded that the anti-NS2 antibodies detected the NS2 antigens with a high level of specificity.

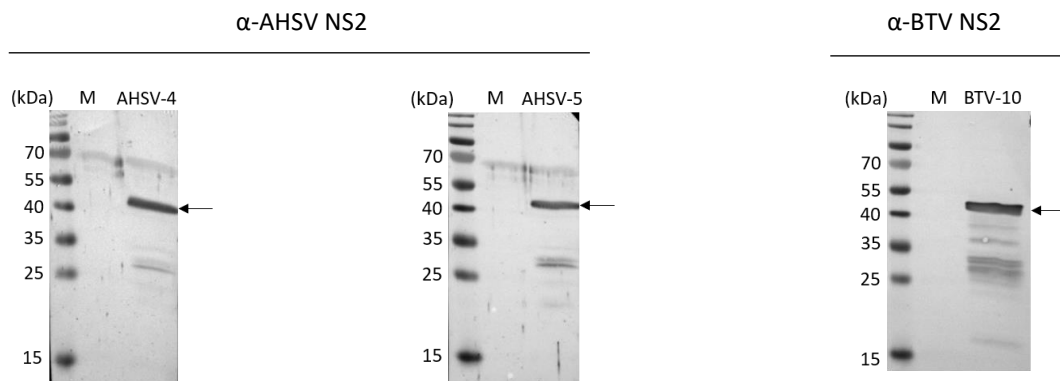


Fig. 2.1. Western blot images showing the expression of NS2 and specificity of detection with available serums. Mock-infected (M) and AHSV-4, AHSV-5 and BTV-10 infected whole cell lysates were separated by SDS-PAGE and immunoblotted for either AHSV or BTV NS2, and revealed bands of expected sizes, 42 kDa (arrows). α = anti.

The distribution and morphology of the VIBs were first characterised and compared between two different serotypes of AHSV and BTV at a single post-infection time point. BSR-T7 cells were infected with AHSV-4, AHSV-5 or BTV-10 and fixed at 24 h pi. Cells were labelled with anti-AHSV or anti-BTV NS2 serum and analysed via confocal laser scanning microscopy (CLSM) (Fig. 2.2).

NS2 showed a strictly cytoplasmic distribution in cells infected with AHSV-4, AHSV-5 or BTV-10. Mock-infected cells labelled with either anti-AHSV or BTV-10 NS2 showed no background labelling, confirming the absence of non-specific binding by the antisera. In the virus-infected cells, most cells showed the distribution of NS2 both homogeneously at a low intensity and in the formation of large, round to oval-shaped structures of high intensity, the latter representing the VIBs. No noticeable differences regarding the distributional or morphological characteristics of the VIBs were observed between the different viruses.

Interestingly, in cells fixed either with PFA (e.g. Fig. 2.2) or methanol-acetone (e.g. BacNS2 sample in Fig. 2.6) and immunolabelled for NS2, the larger VIBs often displayed ring-like appearances, with a higher NS2 intensity at the perimeters of the VIBs, and a lower intensity towards the middle. Small VIBs mostly showed a strong homogeneous fluorescence throughout. When comparing this labelling pattern to published TEM images of the VIBs (Brookes *et al.* 1993; Uitenweerde *et al.* 1995) which showed an even distribution of NS2 throughout the entire section of the VIBs, it is speculated that the ring-like appearances of the VIBs is simply due to the NS2 antibody being hindered from binding to epitopes within the dense protein matrix of aggregated NS2 rather than indicating a differential distribution of NS2 within the VIBs.

NS2

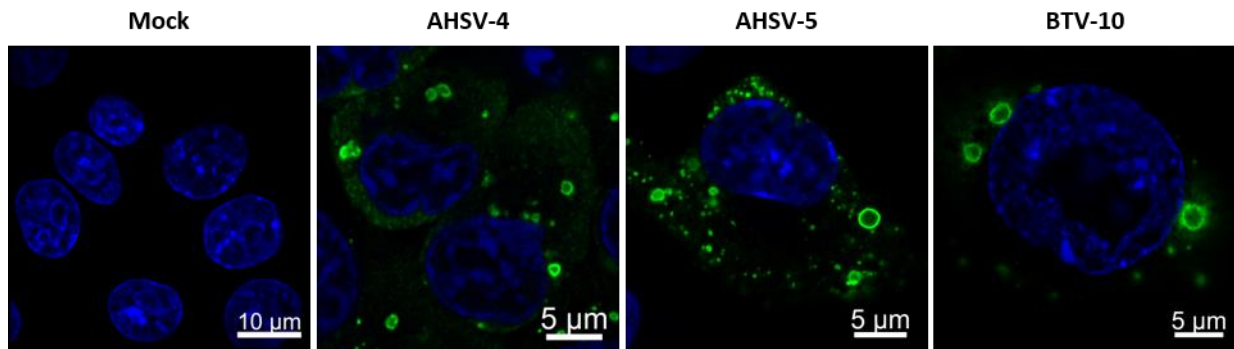


Fig. 2.2. Confocal microscopy images showing the distribution pattern and morphology of the VIBs in virus-infected cells. BSR-T7 cells were mock-infected or infected with AHSV-4, AHSV-5 or BTV-10 and fixed at 24 h pi. Cells were immunolabelled with anti-NS2 primary and anti-rabbit Alexa Fluor 488 secondary antibodies (green); nuclei stained with DAPI (blue). Sizes of scale bars as indicated.

Next, the development of the AHSV VIBs was characterised at different times after infection. BSR-T7 cells were infected with AHSV-4 and fixed at different post-infection time points between 4 and 48 h pi. Cells were subjected to immunolabelling for AHSV NS2 and slides from the different time points were analysed via CLSM.

The axial resolution of a CLSM is approximately 500 nm (Schermelleh *et al.* 2010), and as such the information contained in a single confocal micrograph significantly underrepresents the total cellular content. Z-stack imaging creates micrographs at different focal planes representing the total cell volume. For the purpose of determining the abundance and morphology of all the VIBs in cells at specific time points post-infection, Z-stack images were taken (Fig. 2.3 A). All the individual Z-stack images were then overlaid to produce a maximum intensity projection (MIP), a two-dimensional representation of the data from the total cell volume (Fig. 2.3 B).

At 4 h pi, VIBs were mostly in the form of small, punctate foci, with a few large VIBs detected (Fig. 2.3 C). The overall expression of NS2 increased as post-infection time points advanced, as seen by both the higher intensity in homogeneously distributed NS2 and the formation of more VIBs. In addition, the VIBs appeared larger in size with progression of time, the biggest difference observed between 8 and 12 h pi. There seemed to be a size limit to the VIBs, as their sizes did not increase after a certain time point.

The NS2 foci or VIBs were manually counted in 18 cells at each time point post-infection, and surface areas measured using Zen Blue software. The sizes of the VIBs ranged from 0,1 to 2,6 μm^2 at 4 h pi and 0,1 to 7,7 μm^2 at 48 h pi. Using the data, the VIBs were grouped into three size categories, i.e. small, medium and large (not shown). The large VIBs with a surface area equal to or larger than 1 μm^2 showed most marked differences in numbers between the different time points, and were thus the focus of analyses. The number of large VIBs seemed to have increased linearly until 24 h pi, after which they plateaued until 48 h pi (Fig. 2.3 D). From 36 h pi, a large extent of cell death had occurred (not shown). From this data, it could be concluded that 24 h pi

is the time point where large VIBs are the most abundant, and the cell viability is not yet compromised by the infection.

The accumulation of intracellular NS2 over time was confirmed by immunoblotting (Fig. 2.3. E). BSR-T7 cells were infected with AHSV-5 and harvested at various post-infection time points between 6 and 24 h pi. Mock-infected BSR-T7 cells were included as controls. Samples were subjected to Western Blotting for AHSV NS2, using anti-actin as a loading control. The band intensities of NS2 at the different post-infection time points were quantified relative to the mock band. NS2 protein was first detected on Western blot from 12 h pi onwards, with the intensities of the bands representing NS2 increasing with progression of infection. The bands representing actin remained relatively constant, confirming consistent sample loading volumes.

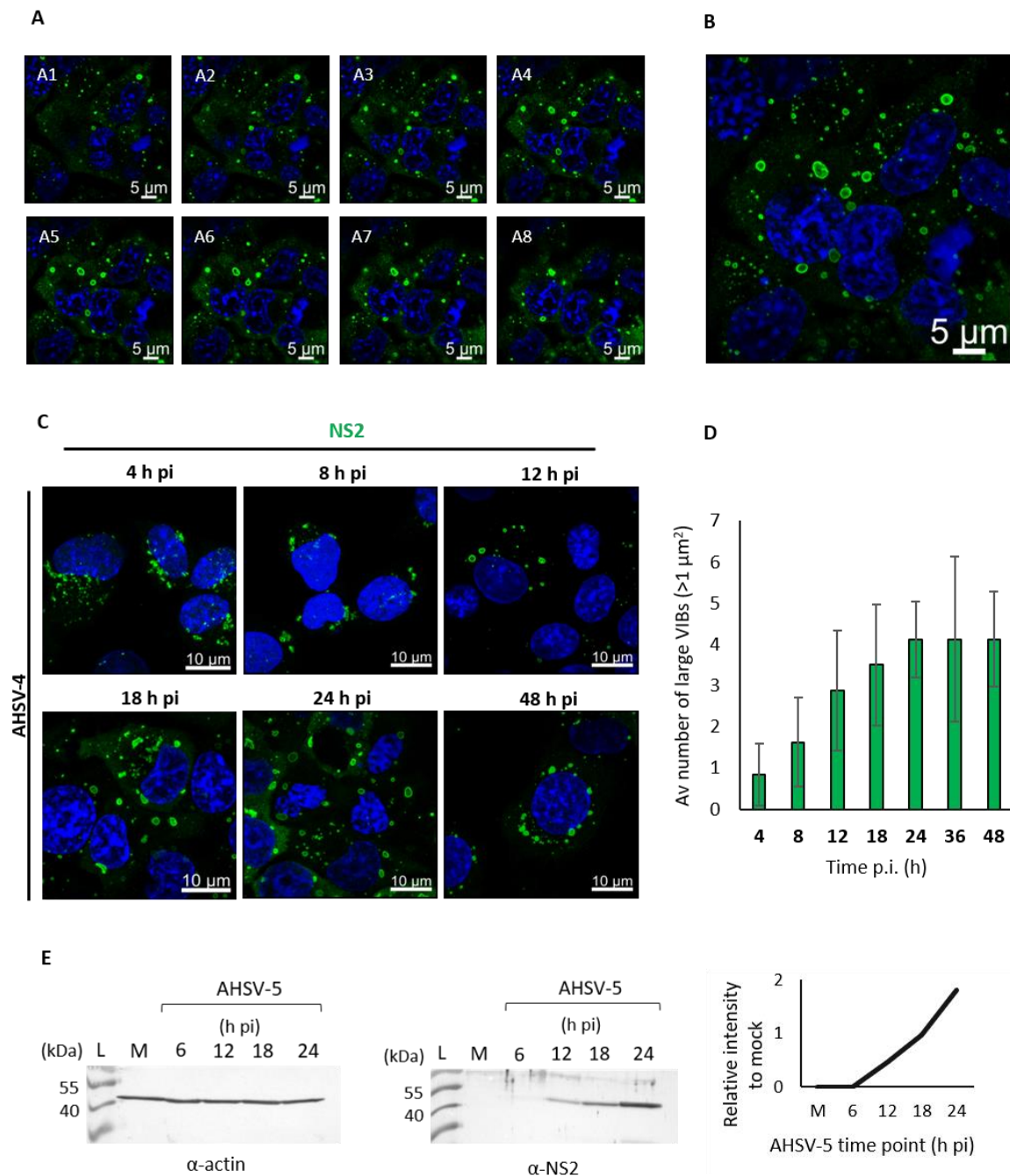


Fig. 2.3. Confocal microscopy images showing the characterisation of VIBs at different stages of AHSV infection. AHSV-4-infected BSR-T7 cells were immunolabelled with anti-AHSV NS2 primary and anti-rabbit Alexa Fluor 488 secondary antibodies (green); nuclei stained with DAPI (blue). Sizes of scale bars as indicated, showing (A) Z-stack images of an infected cell at 24 h pi, to detect VIBs at different layers (A1=bottom; A8=top) of the cell; (B) the MIP image resulting from merging the Z-stack images in (A), showing all the VIBs in the cell volume in a single view; (C) MIP images at different time points post-infection; (D) graph showing the quantification of the number of large VIBs as infection progressed. Error bars= SD of the mean; Av= average; h pi= hours post-infection; (E) Mock-infected (M) and AHSV-5 infected BSR-T7 cell lysates were separated by SDS-PAGE and subjected to immunoblotting for actin or NS2, graph to the right shows intensity of NS2 at different hours post-infection with AHSV-5 relative to mock. Sizes of the marker as indicated.

2.3.1.2. *The formation of VIBs is independent of other AHSV proteins and solely mediated by NS2*

It is known that for AHSV and BTV the formation of VIB-like structures is solely dependent on the expression of NS2 (Thomas *et al.* 1990; Uitenweerde *et al.* 1995). Two systems were available to express AHSV NS2 outside the context of an AHSV infection, namely a plasmid-based transient mammalian expression system (phCMV-NS2) and a recombinant baculovirus expression system (BacNS2). The efficacy of these systems in expressing NS2 and forming VIB-like structures was first established.

The phCMV-NS2 plasmid consisted of the open reading frame of AHSV Seg-8 under control of a CMV promoter. The BacNS2 recombinant virus consisted of Seg-8 of AHSV-9 under control of a late polyhedron promoter (Uitenweerde *et al.* 1995b). BacNS2 was amplified in Sf9 cells and harvested when complete CPE was observed.

The size of the phCMV-NS2 plasmid was confirmed with HindIII restriction enzyme digestion followed by agarose gel electrophoresis (Fig. 2.4). Undigested (U) plasmids showed the different conformations, and digested (D) plasmids showed the linear plasmid conformation of expected size, 3661 bp, the sum of Seg-8 (1103 bp) and the phCMV vector sequence (2558 bp).

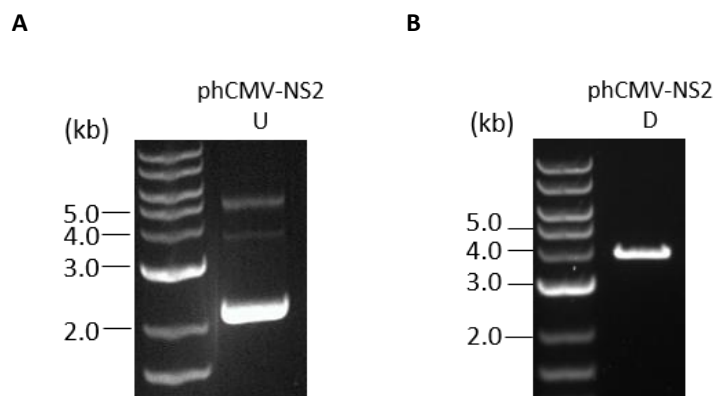


Fig. 2.4. Agarose gel electrophoresis images of phCMV-NS2 plasmid without (A) and after (B) restriction enzyme digestion with HindIII, showing the expected size of the 3661 bp plasmid. U= undigested; D= RE digested. Sizes of the marker as indicated.

To confirm the expression of AHSV NS2 from the available expression systems, BSR-T7 cells were transfected with phCMV-NS2, and Sf9 cells were infected with BacNS2. Cells were harvested at 48 hours post-transfection (h pt) or 48 h pi, and samples were subjected to SDS-PAGE separation followed by immunoblotting with anti-AHSV NS2 serum (Fig. 2.5). Mock-transfected BSR-T7 cells, mock-infected Sf9 cells and AHSV-4-infected BSR-T7 cells were used as controls. Both phCMV-NS2-transfected and BacNS2-infected cells showed a clear band of 42 kDa in size similar to what is expressed in AHSV-4-infected cells. This band was absent from or faint in

the mock samples, and minimal background was detected. This showed that both the expression systems expressed NS2 of the correct size, which could be accurately detected with an anti-AHSV NS2 serum.

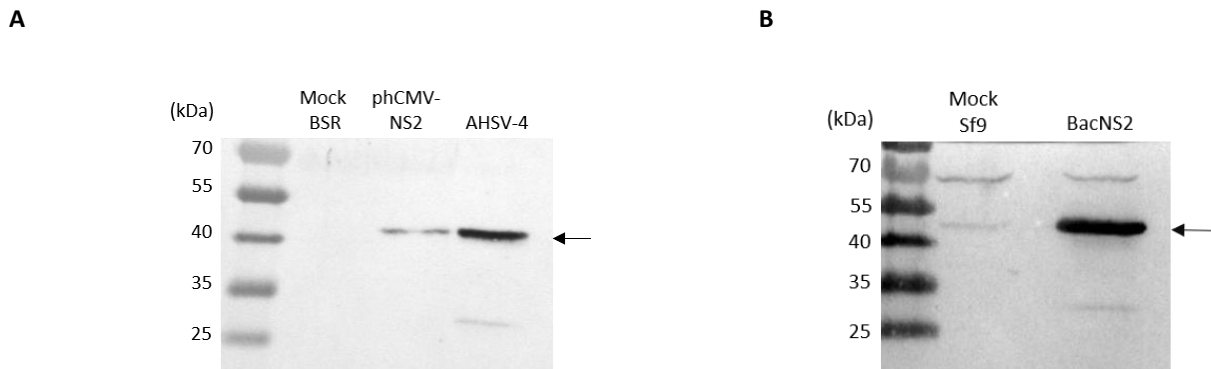


Fig. 2.5. Western blots showing the expression of 42 kDa NS2 (arrow) from both expression systems. Cell lysates from (A) BSR-T7 cells mock-transfected or transfected with phCMV-NS2 or infected with AHSV-4 and (B) Sf9 cells mock-infected or infected with BacNS2 were separated by SDS-PAGE and immunoblotted with anti-AHSV NS2 serum.

Next, the distribution and morphology of NS2 or VIB-like structures formed by phCMV-NS2 and BacNS2 were characterised. Sf9 cells were infected with BacNS2, and BSR-T7 cells transfected with phCMV-NS2. Sf9 cells were fixed (methanol-acetone) at 48 h pi and BSR-T7 cells were fixed at 48 h pt. Cells were labelled with anti-AHSV NS2 and analysed via CLSM.

In phCMV-NS2 transfected BSR-T7 cells, NS2 mostly distributed as small punctate foci in the cytoplasm (Fig. 2.6.). In some cases, larger aggregates of NS2 were formed that were similar in shape but usually appeared to be slightly smaller in size than VIBs formed during an AHSV infection. Although the larger VIB-like structures mostly resembled AHSV VIBs in shape, some showed a less rounded and more amorphous morphology. In BacNS2-infected Sf9 cells, NS2 presented both as a disperse cytoplasmic distribution and in the formation of small foci as well as large VIB-like structures. Here, the VIB-like structures sometimes had a more elongated shape, probably influenced by the limited size of the Sf9 cytoplasmic volume. Baculoviruses replicate in the nucleus, resulting in nuclear enlargement.

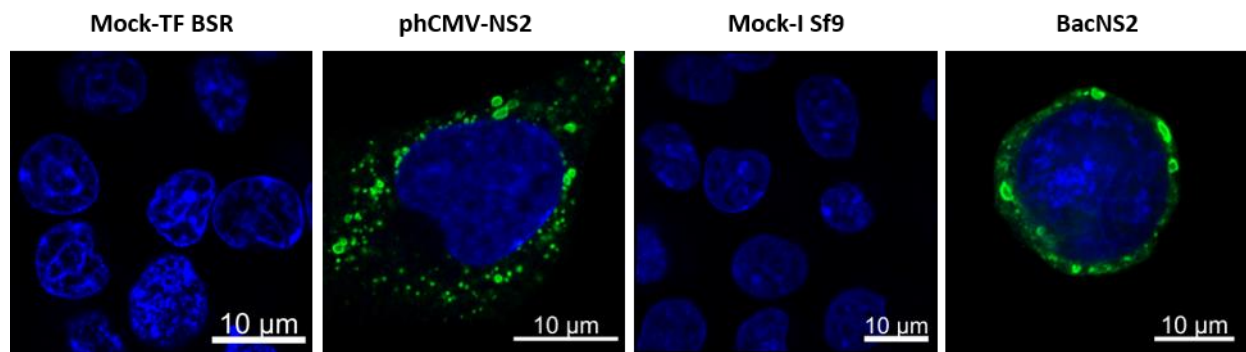


Fig. 2.6. Confocal microscopy images showing the distribution pattern of NS2 and morphology of VIB-like structures when NS2 is expressed recombinantly. BSR-T7 cells were transfected with phCMV-NS2 and Sf9 cells were infected with BacNS2. Cells were fixed at 48 h pt or 48 h pi and immunolabelled with anti-AHSV NS2 primary and anti-rabbit Alexa Fluor 488 secondary antibodies (green); nuclei stained with DAPI (blue). Mock-TF= Mock-transfected; Mock-I= mock-infected. Sizes of scale bars as indicated.

2.3.1.3. *The coalescence of highly mobile NS2 foci can be detected in live BSR-T7 cells*

Until now, the characterisation of AHSV VIBs has been restricted to fixed, non-living cells. In this section, we aimed to elucidate in real-time how VIBs are formed. This was done by live-cell analyses of BSR-T7 cells transfected with a plasmid expressing an NS2-eGFP fusion protein (phCMV-NS2-eGFP). This allowed NS2 to be tracked in live cells without requiring fixation or labelling.

The construction of the phCMV-NS2-eGFP plasmid is outlined in Fig. 2.7. The phCMV-NS2 plasmid was used as backbone vector, for insertion of an eGFP downstream of the NS2 open reading frame (ORF). The source of the eGFP sequence to be inserted was the phCMV-NS4-eGFP plasmid. To preserve native folding of NS2 and eGFP after being conjugated, a serine-glycine (S-G) linker, found between the NS4 and eGFP ORFs, was included in the cloning reaction. In brief, primers were designed specifically for the vector and insert fragments and contained homologous 5' overhangs to allow vector-insert annealing. The vector-specific primer was designed to remove the stop codon from the NS2 ORF. The plasmids were PCR amplified and linearised using the specific primers. After the sizes of the amplicons had been confirmed with agarose gel electrophoresis, the reactions were subjected to PCR clean-up. The vector and insert fragments were ultimately joined with an In-Fusion cloning reaction.

The PCR-linearised phCMV-NS2 vector and eGFP insert fragments showed single bands of expected sizes, 3661 bp for phCMV-NS2 vector and 763 bp for eGFP with the S-G linker (Fig. 2.8 A and B). A small amount of background was observed in the negative lane of phCMV-NS2, which might have been due to cross-contamination of template in the negative reaction. After PCR clean-up, the linearised fragments showed the same size and adequate yields of DNA (Fig. 2.8 C). The phCMV-NS2 backbone vector contains two EcoRI restriction endonuclease sites, at positions 1816 and 4386. The phCMV-NS2-eGFP construct obtained after

In-Fusion cloning showed the expected size of a total of 4393 bp after restriction enzyme digestion with EcoRI, representing the combined sizes of phCMV-NS2 and eGFP with the serine-glycine linker (Fig. 2.8 D). This confirmed successful joining of the fragments, and construction of the phCMV-NS2-eGFP plasmid.

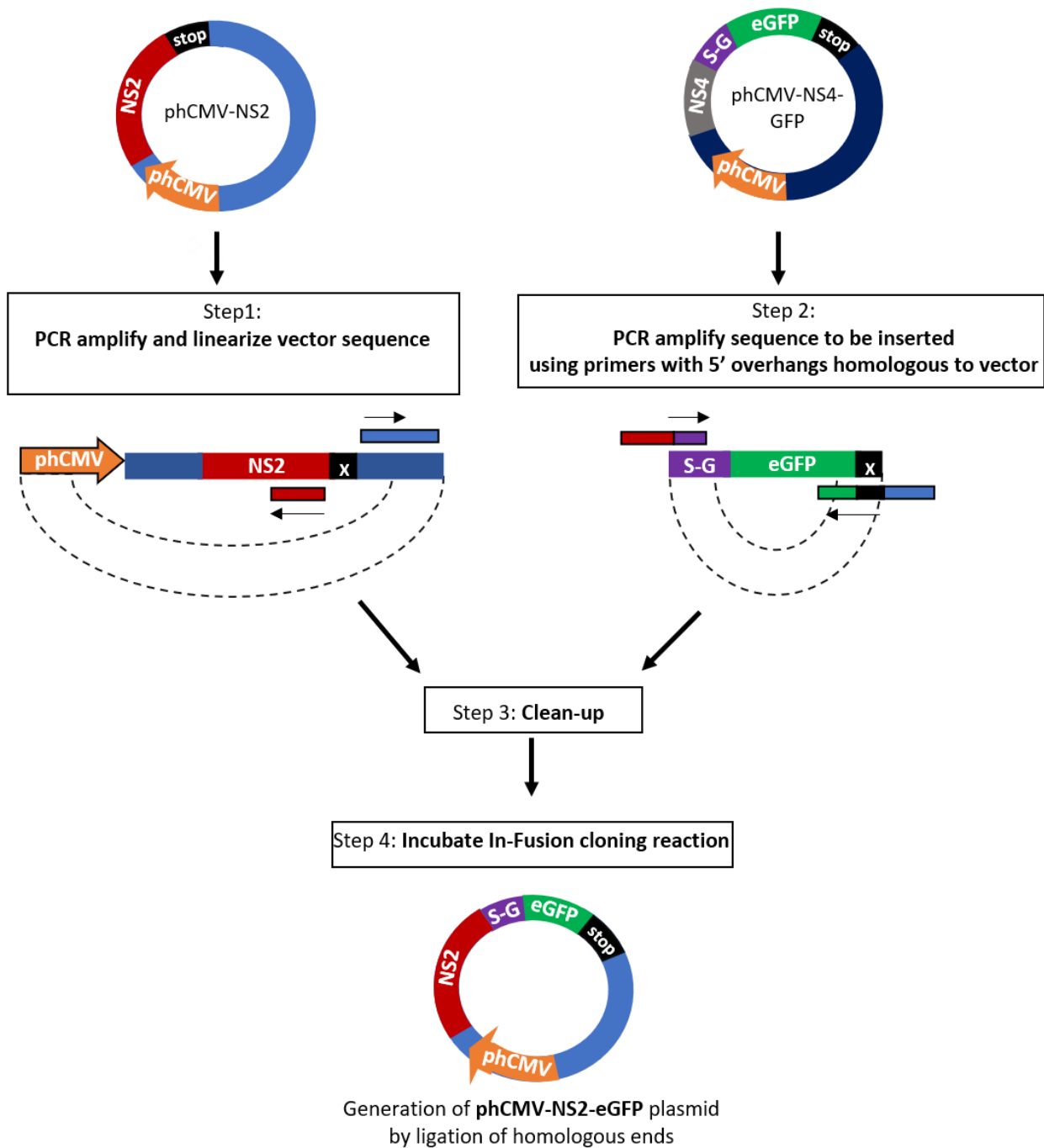


Fig. 2.7. A schematic diagram explaining the construction of the phCMV-NS2-eGFP plasmid using the In-Fusion HD cloning kit. The specific primers used are represented by the rectangles, their colours corresponding to the site of annealing, and arrows indicating the directions of priming. X=stop codon.

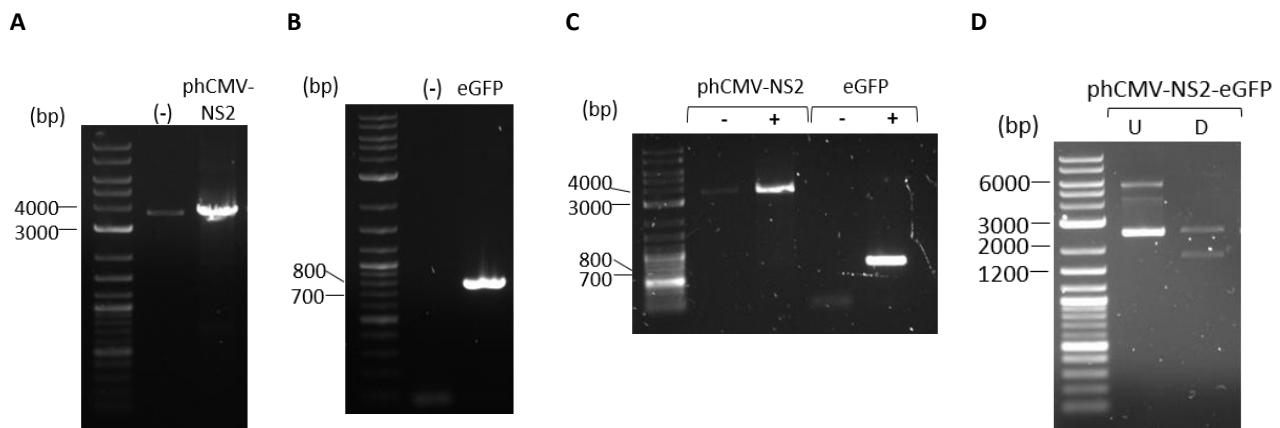


Fig. 2.8. Agarose gel electrophoresis images of (A) linearised phCMV-NS2 and (B) linearised eGFP fragments, (C) following PCR clean-up; (D) the resulting phCMV-NS2-eGFP plasmid in the undigested and EcoRI-digested form, confirming the size of the 4438 bp construct. (-)= negative reactions without template; (+)= template reactions; U= undigested; D= restriction enzyme digested. Sizes of the markers as indicated.

To verify the expression of the NS2-eGFP fusion protein, BSR-T7 cells were transfected with phCMV-NS2-eGFP and whole cell lysates were subjected to Western blotting using an anti-AHSV NS2 serum. Cell lysates from phCMV-NS2 and pCMV-eGFP transfected cells immunoblotted for NS2 or eGFP were used as controls. Lysates from pCMV-eGFP transfected cells blotted for eGFP showed the expected size of 27 kDa (Fig. 2.9 A). Both the positive controls for NS2, namely AHSV-4 infected and phCMV-NS2 transfected lysates showed a 42 kDa band on immunoblots for NS2 (Fig. 2.9 B). Lysates from phCMV-NS2-eGFP transfected cells showed a faint band of around 70 kDa, which is expected as the combined molecular weight of the fusion protein is 69 kDa, from 27 kDa (eGFP) and 42 kDa (NS2) (Fig. 2.9 A and B). The low band intensity is probably due to low transfection efficiency, as also seen by the low intensity band of phCMV-NS2 cell lysate.

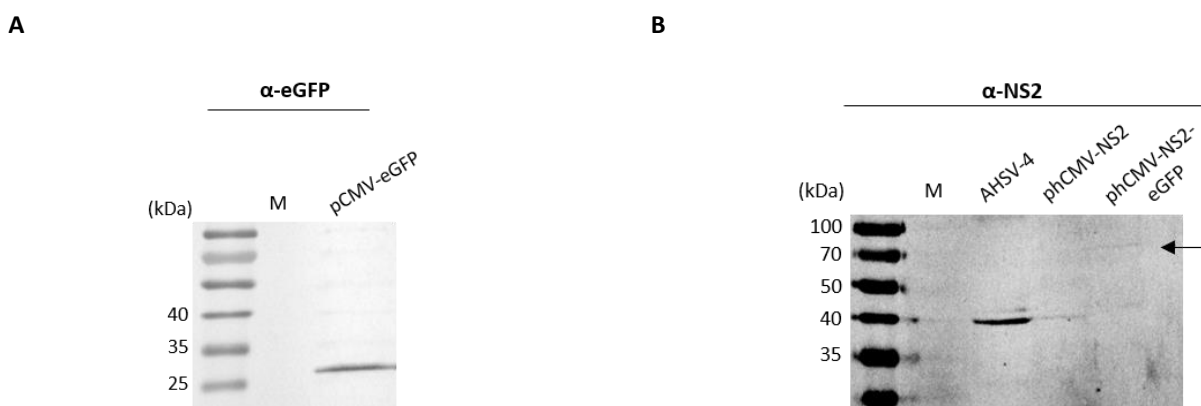


Fig. 2.9. Western blot images showing (A) the expression of eGFP (27 kDa); (B) the expression of a 69 kDa NS2-eGFP protein (arrow). Whole cell lysates of BSR-T7 cells transfected with pCMV-eGFP, phCMV-NS2, or phCMV-NS2-eGFP, or infected with AHSV-4 were separated by SDS-PAGE and immunoblotted for eGFP or NS2. α= anti; M=Mock-infected. Sizes of the marker as indicated.

The intracellular distribution and morphology of the NS2-eGFP fusion protein was characterised. BSR-T7 cells were transfected with pHCMV-NS2-eGFP and fixed at 48 h pt. Cells were analysed with CLSM, using cells transfected with pHCMV-NS2 (labelled for AHSV NS2) and pCMV-eGFP as controls. Compared to the homogenous cytoplasmic and nuclear distribution displayed by eGFP, the distribution of NS2-eGFP was restricted to the cytoplasm (Fig. 2.10). NS2-eGFP formed an abundance of punctate foci, as well as larger spherical-shaped structures similar to those seen in pHCMV-NS2-transfected cells. This confirms the validity of this system to be utilised as a tool for characterising VIB-like structures when NS2 is expressed in both the absence of other virus proteins and NS2 labelling.

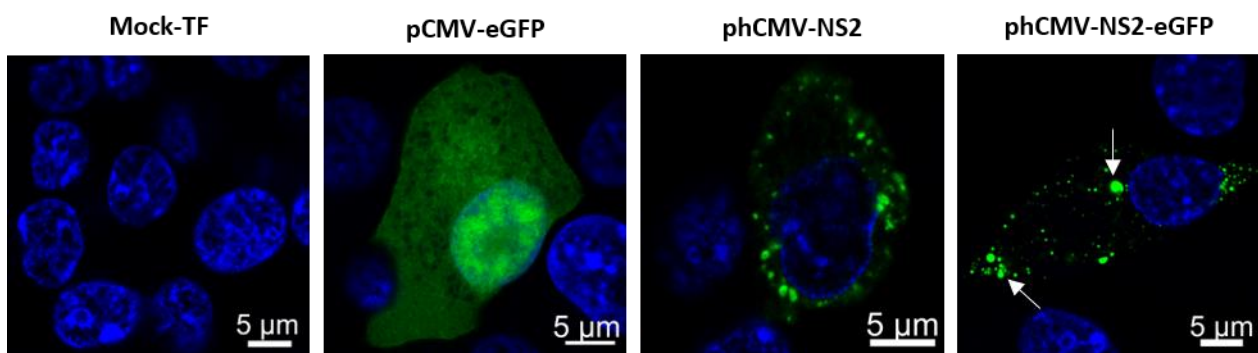


Fig. 2.10. Confocal microscopy images showing the intracellular distribution pattern of NS2-eGFP fusion protein compared to individually expressed NS2 and eGFP. BSR-T7 cells were either transfected with pHCMV-NS2 and immunolabelled with anti- AHSV NS2 primary and anti-rabbit Alexa Fluor 488 secondary antibodies (green), or transfected with pCMV-eGFP or pHCMV-NS2-eGFP and not labelled; nuclei stained with DAPI (blue). Mock-TF= mock-transfected. Arrows indicate the formation of VIB-like structures in pHCMV-NS2-eGFP transfected cells. Sizes of scale bars as indicated.

As an additional confirmation that the fluorescent NS2-eGFP foci represented all the NS2 protein that was being expressed following transfection, it was decided to also fix and label these cells with anti-NS2 serum, and to detect the anti-NS2 with a red fluorescing secondary antibody. BSR-T7 cells were transfected with pHCMV-NS2-eGFP, fixed at 48 h pt, and labelled with anti-AHSV NS2 and anti-rabbit Alexa Fluor 594. Cells were analysed via CLSM and visualised in the green and the red channel (Fig. 2.11).

The fluorescent NS2-eGFP foci also labelled positive for NS2 in each instance. This confirms the efficacy of this system to be used as a mimic of native NS2 expression to characterise VIB-like structures in the absence of labelling. Interestingly, where the large NS2-eGFP VIB-like structures showed an even distribution of eGFP fluorescent signal, NS2 often labelled only at the margins, giving rise to ring-like appearances. This further supports the hypothesis that the decrease in signal intensity towards the central regions of the VIBs or VIB-like structures seen with NS2 labelling is technical in nature, possibly due to epitope hindering of the NS2 antigens within the dense matrix of the VIBs.

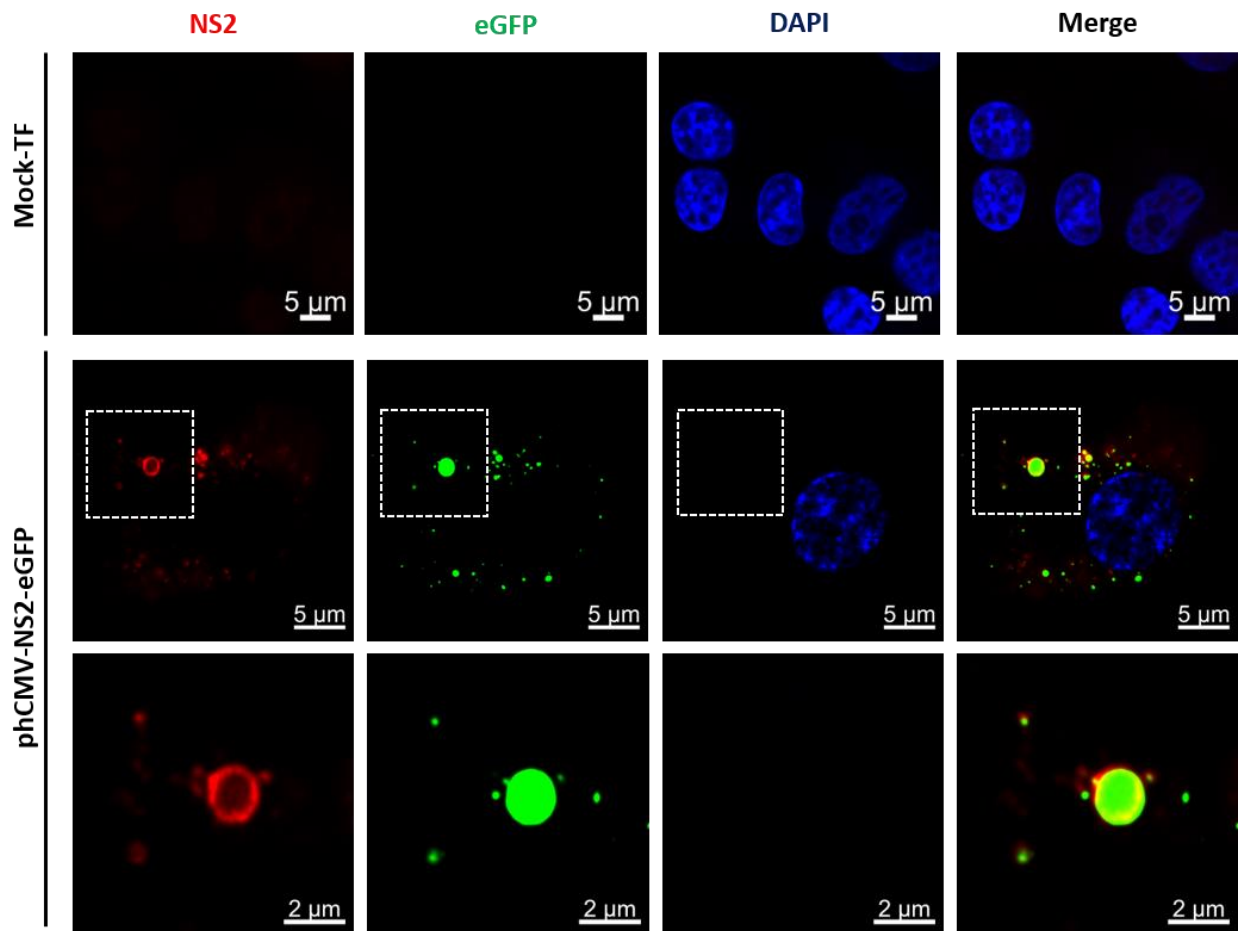


Fig. 2.11. Confocal microscopy images showing NS2 labelling and eGFP fluorescence of NS2-eGFP fusion proteins. BSR-T7 cells were transfected with phCMV-NS2-eGFP and fixed at 48 h pt. Cells in the left panel were immunolabelled with anti-AHSV NS2 and Alexa Fluor 594 (red); the second panel shows eGFP fluorescence (green); the third panel shows nuclei stained with DAPI (blue). The images in the bottom panel are higher magnification views of the areas demarcated by the white box in images in the middle row. Mock-TF= mock-transfected. Sizes of scale bars as indicated.

It was aimed to characterise the dynamic behaviour of NS2 foci, with the aim of elucidating how they change from a dispersed distribution to forming larger inclusions or VIB-like structures. Live-cell imaging was conducted using BSR-T7 cells transfected with phCMV-NS2-eGFP. From 6 h pt, cells were kept at ideal living conditions (37°C, 5% CO₂ and humidity). Cells were selected for analysis which appeared to be at an early stage of NS2-eGFP expression, i.e. showing a few small punctate foci of relatively low eGFP intensity initially. The cells were monitored over a 72-hour period, taking an image every 2 min with the CLSM. The snapshots were then compared between the different time frames to elucidate the pattern of distribution of NS2-eGFP foci over time.

A video of the live cell results can be accessed by following this link: <https://youtu.be/gUJEUy8cBdo>. During the first few hours of expression, small punctate NS2-eGFP foci increased in numbers (Fig. 2.12 a-c), and

moved around rapidly and directionless (Fig. 2.12 d-f). Once adjacent NS2-eGFP foci were in close proximity to one another, their erratic movement declined and they appeared to coalesce, the foci remaining in close association with one another (Fig. 2.12 g-i). This all occurred within a 10-hour period, after which cell death often occurred. The data obtained thus far provides a preliminary explanation to how NS2 punctate foci distribute and behave to form the precursors of the VIBs.

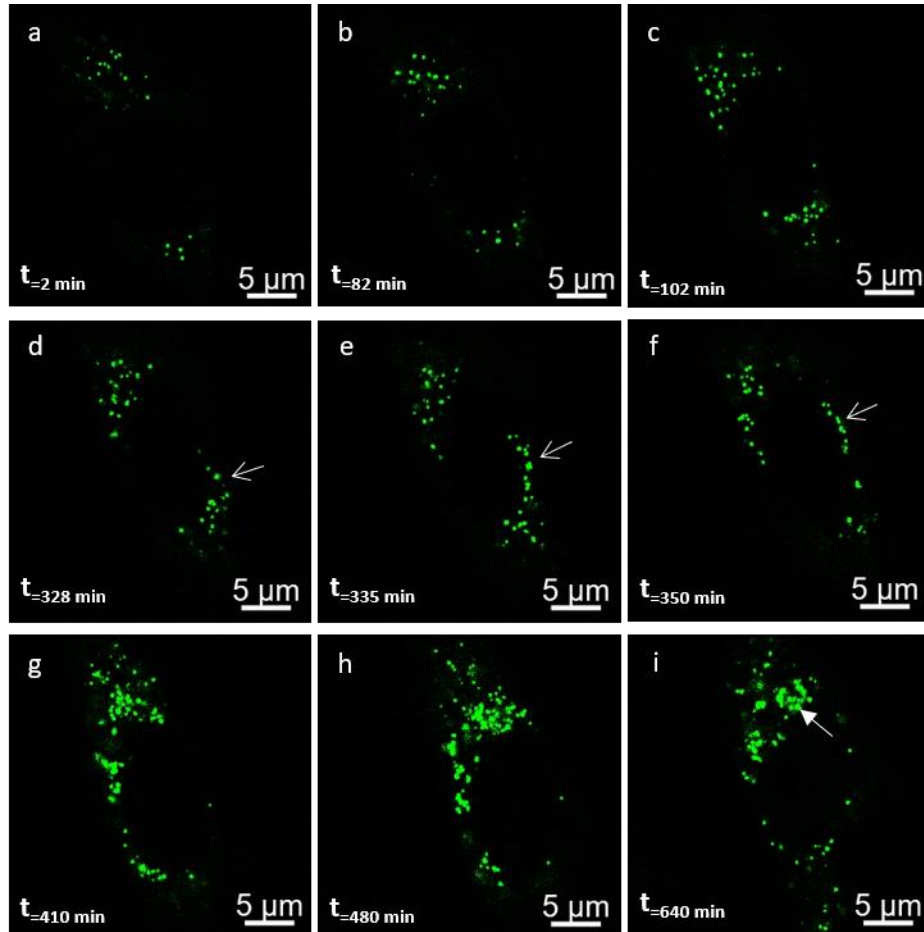


Fig. 2.12. Individual confocal micrographs from live-cell imaging showing the aggregation process of NS2 foci to form the precursors of the VIBs. BSR-T7 cells were transfected with phCMV-NS2-eGFP and analysed via live-cell imaging from 6 h pt. Green signal shows NS2-eGFP fluorescence, the times (t) at which images were taken, progress from (a) to (i), and are indicated to the bottom left in each image. Open arrows in (d) to (f) indicate the change in position of a number of NS2 foci; solid arrow (i) indicates a larger cluster of NS2 foci or a VIB. Sizes of scale bars as indicated.

The results obtained up to this point described the morphology of the VIBs to be similar between two different serotypes of AHSV as well as BTV. The observed characteristics of the VIBs at different post-infection time points allowed determining the optimal post-infection time point for downstream analyses of the VIBs. Controls for assessing the VIB-like structures when NS2 is expressed recombinantly in the absence of other AHSV proteins were also established. These results showed the first characterisation of AHSV NS2 foci in live cells and provide a preliminary description of the events leading to VIB formation. Next, it was explored whether the VIBs have an additional function to what is already known.

2.3.2. Actively translating ribosomes localise preferentially to AHSV VIBs

2.3.2.1. Puromycin-incorporated polypeptides localise to AHSV VIBs

Thus far, the sole function of the VIBs has been described as being factories wherein core particle assembly and genome replication occur (Brookes *et al.* 1993; Kar *et al.* 2007). In this section, it was explored whether the VIBs of AHSV have additional roles by becoming sites for virus protein synthesis.

The ribopuromylation (RPM) assay is a biochemical method that has been developed to label and detect ribosomes that are actively involved in protein synthesis (David *et al.* 2012). This method was successfully used to detect active translation in reovirus virus factories (Desmet *et al.* 2014). The RPM assay is outlined in Fig. 2.13. Briefly, cells are pre-treated with a chemical inhibitor, emetine, which stalls ribosome-mRNA complexes that are in the process of translation elongation. An antibiotic and t-RNA analogue, puromycin (PMY), is then transiently added, which enters the A site of the ribosome and forms a covalent bond with the C-terminus of the nascent polypeptide chain, halting further translation elongation (Pestka 1971). PMY molecules, which in effect represent actively translating ribosomes, are ultimately detected with immunolabelling.

To visualise active translation in orbivirus-infected cells, BSR-T7 cells were infected with AHSV-4, AHSV-5 or BTV-10. At 24 h pi, cells were subjected to the RPM assay, fixed and dual labelled with anti-puromycin primary (with anti-mouse Alexa Fluor 594 secondary antibody), and anti-AHSV NS2 primary (with anti-rabbit Alexa Fluor 488 secondary antibody). Mock-infected cells and virus-infected cells pre-treated with emetine without the addition of PMY (PMY-), were included as controls (Fig. 2.14-2.19).

In PMY+ cells, PMY showed a strong positive labelling that was restricted to the cytoplasm (Fig. 2.14; 2.16 and 2.18). PMY- cells showed no significant PMY signal. PMY- cells infected with either AHSV-4, AHSV-5 or BTV-10 also did not show any detectable levels of PMY, confirming the absence of non-specific antibody cross-interactions. Signal intensity plot profiles provide a tangible approach towards quantifying the relative intensities of two or more fluorophores at any chosen area of a micrograph (signal intensity is presented on the y-axis, corresponding to the physical position along the line drawn on the micrograph which is presented on the x-axis). This corroborated the visual results from the different micrographs. The plot profiles of all PMY- cells showed a negligible level of PMY signal, which increased substantially in all PMY+ cells. This confirmed the specificity of the antibody towards the PMY antigens.

In mock-infected cells, PMY mostly showed a diffuse cytoplasmic distribution. However, in cells infected with either AHSV-4 (Fig. 2.15; 2.16), AHSV-5 (Fig. 2.17; 2.18) or BTV-10 (Fig. 2.19; 2.20), PMY had a diffuse cytoplasmic distribution but also showed the formation of large foci. These foci often had a brighter intensity compared to the surrounding dispersedly distributed PMY signal. Co-labelling for NS2 showed that the PMY in the large foci colocalised with the VIBs. Signal intensity plot profiles confirmed an elevated intensity of

PMY within the VIBs, with a stronger fluorescent intensity than that of the diffuse cytoplasmic PMY signal. The central regions of the VIBs most often coincided with a peak in PMY intensity, even in cases where NS2 only labelled at the VIB margins. The differential patterns in NS2 and PMY labelling eliminate the possibility of non-specific labelling due to antibody cross-interactions.

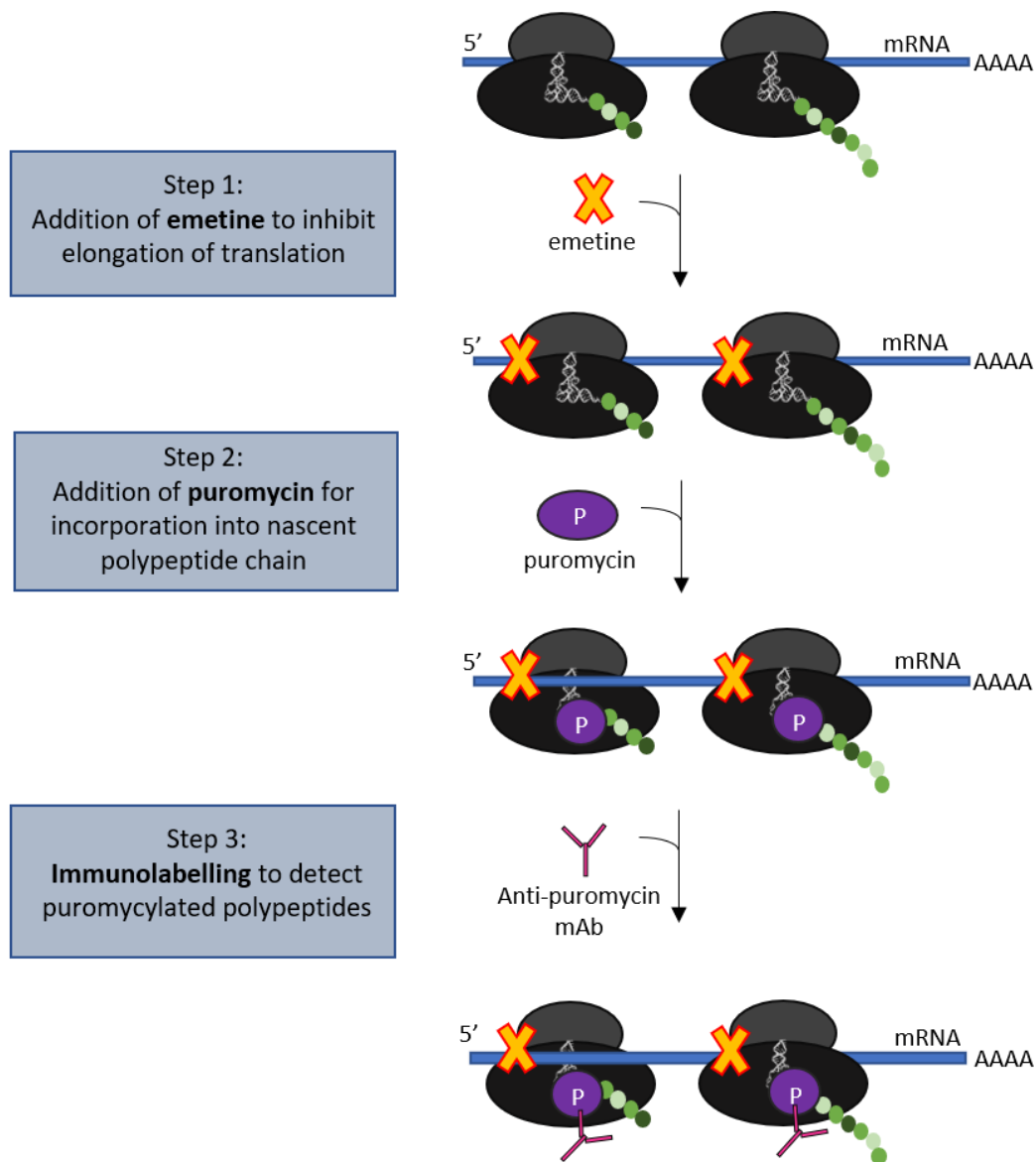


Fig. 2.13. A schematic diagram explaining the ribopuromycylation (RPM) procedure to detect actively translating ribosomes.

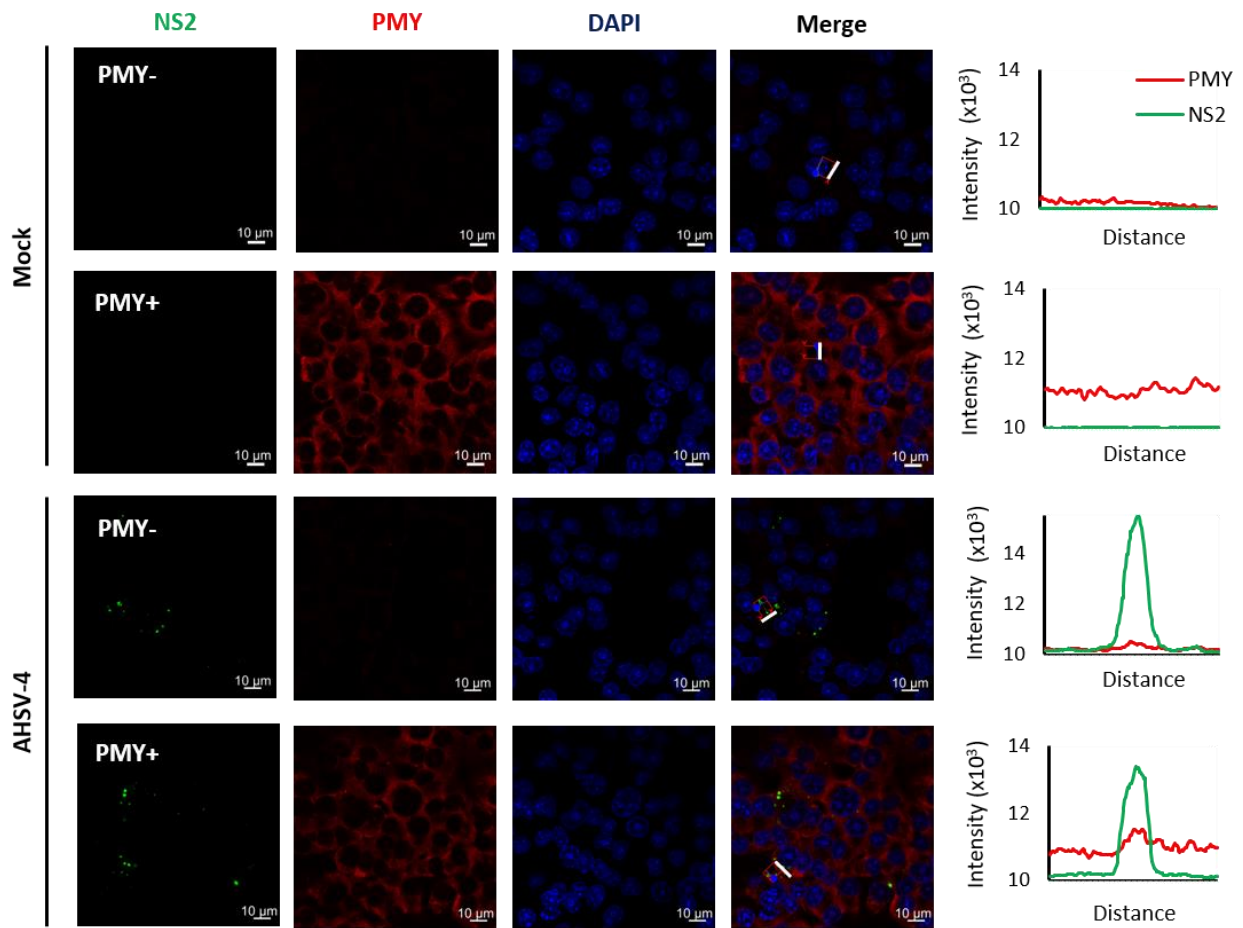


Fig. 2.14. Confocal microscopy images showing the distribution of actively translating ribosomes in mock- and AHSV-4-infected cells. Mock- and AHSV-4-infected BSR-T7 cells were incubated in the absence (PMY-) or presence (PMY+) of puromycin (PMY) at 24 h pi. Cells were immunolabelled with antibodies against NS2 (green) and PMY (red); nuclei stained with DAPI (blue). The plot profiles display the fluorescence intensity values of PMY and NS2 measured along the white lines drawn in the merged images. Sizes of scale bars= 10 μ m.

AHSV-4

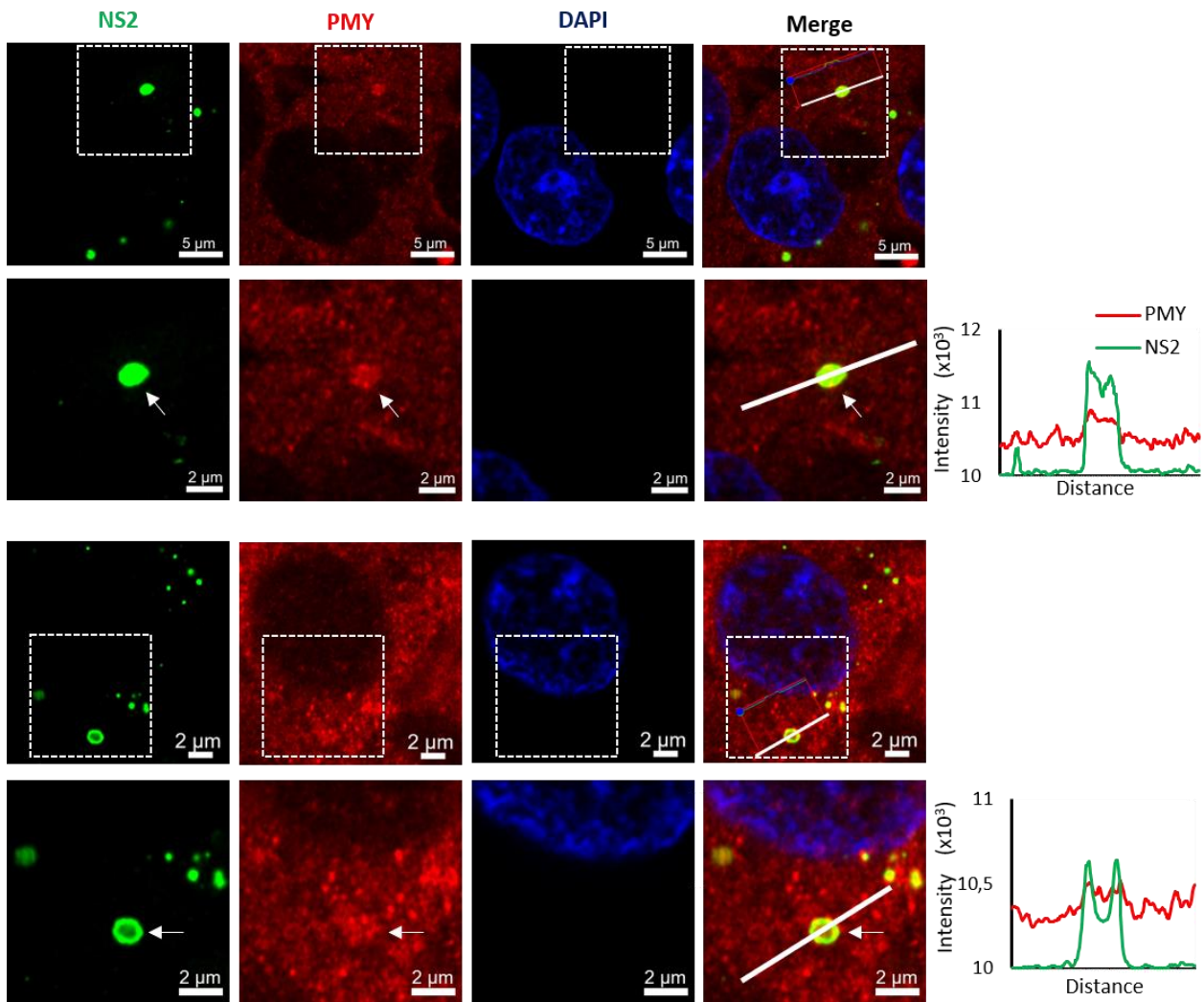


Fig. 2.15. Confocal microscopy images showing the colocalisation of AHSV-4 VIBs with large foci of actively translating ribosomes (arrows). AHSV-4-infected BSR-T7 cells were subjected to the RPM assay, fixed at 24 h pi and immunolabelled with antibodies against NS2 (green) and PMY (red); nuclei stained with DAPI (blue). Each second row shows a higher magnification view of the areas demarcated by the white block in the images above it. The plot profiles display the fluorescence intensity values of PMY and NS2 measured along the white lines drawn in the merged images. Sizes of scale bars as indicated.

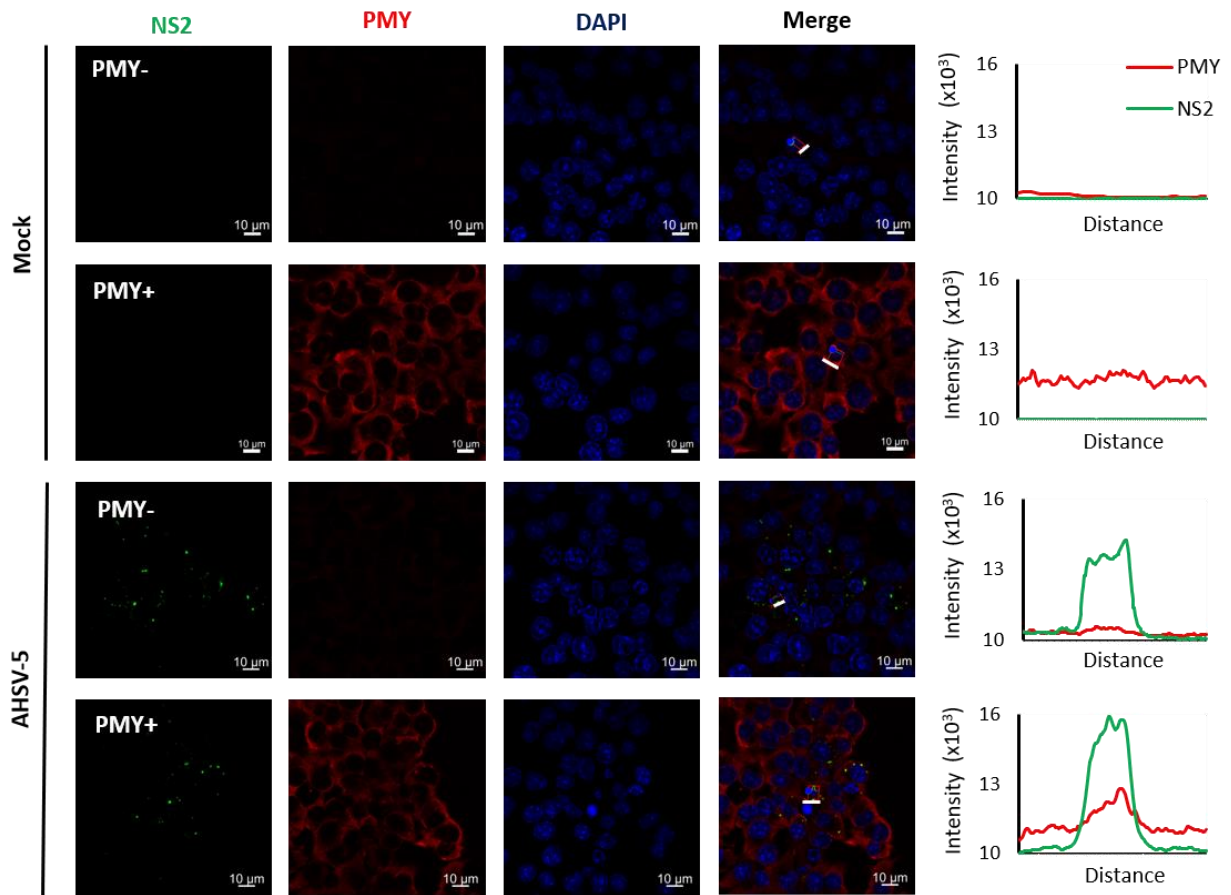


Fig. 2.16. Confocal microscopy images showing the distribution of actively translating ribosomes in mock- and AHSV-5-infected cells. Mock- and AHSV-5-infected BSR-T7 cells were incubated in the absence (PMY-) or presence (PMY+) of puromycin (PMY) at 24 h pi. Cells were immunolabelled with antibodies against NS2 (green) and PMY (red); nuclei stained with DAPI (blue). The plot profiles display the fluorescence intensity values of PMY and NS2 measured along the white lines drawn in the merged images. Sizes of scale bars= 10 μm.

AHSV-5

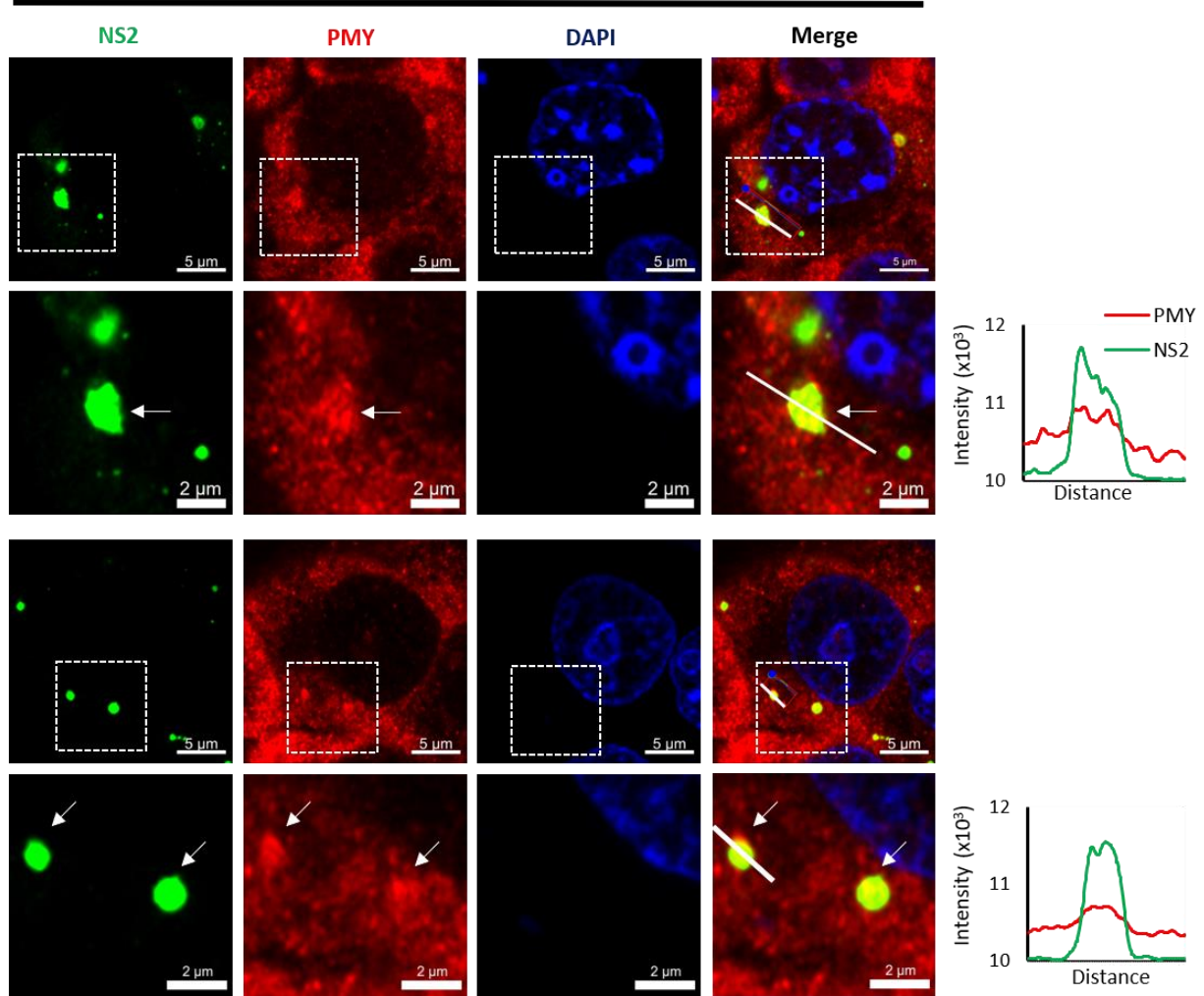


Fig. 2.17. Confocal microscopy images showing the colocalisation of AHSV-5 VIBs with large foci of actively translating ribosomes (arrows). AHSV-5-infected BSR-T7 cells were subjected to the RPM assay, fixed at 24 h pi and immunolabelled with antibodies against NS2 (green) and PMY (red); nuclei stained with DAPI (blue). Each second row shows a higher magnification view of the areas demarcated by the white block in the images above it. The plot profiles display the fluorescence intensity values of PMY and NS2 measured along the white lines drawn in the merged images. Sizes of scale bars as indicated.

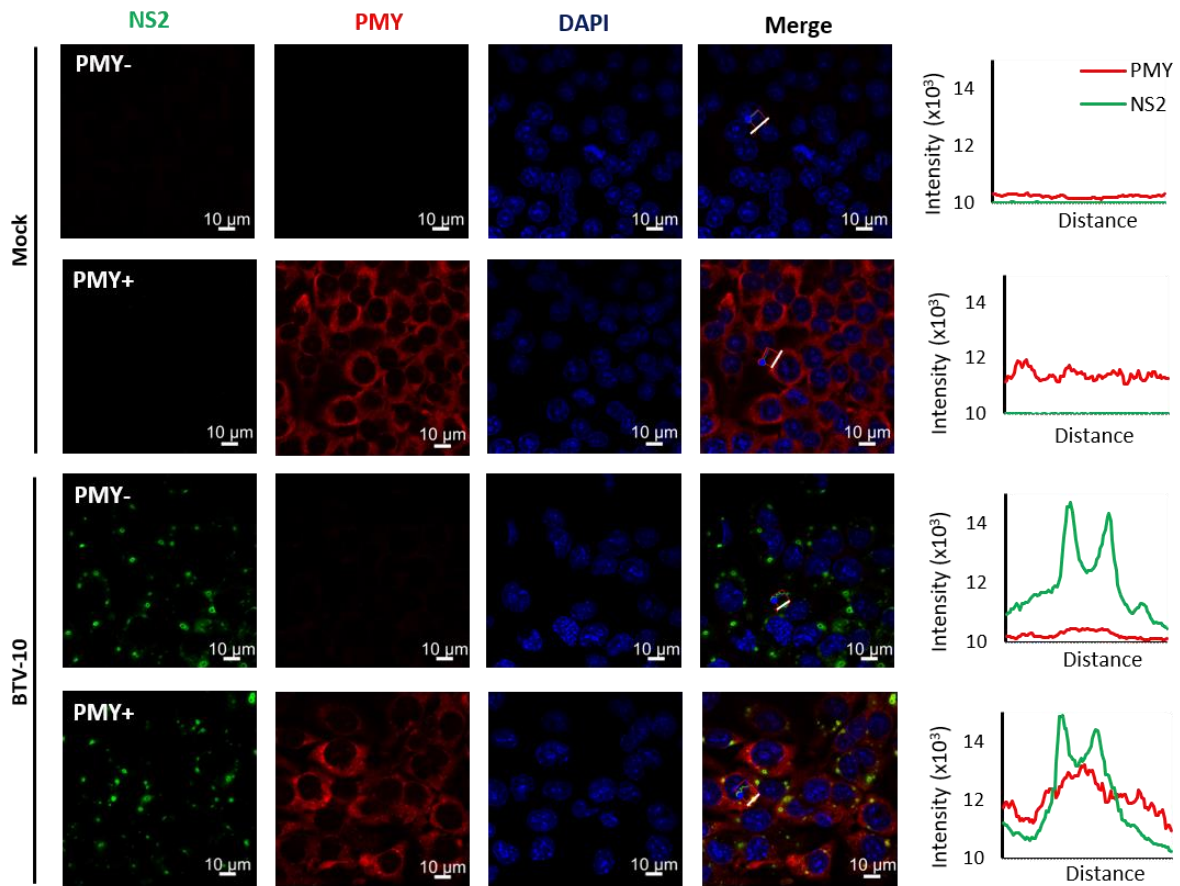


Fig. 2.18. Confocal microscopy images showing the distribution of actively translating ribosomes in mock- and BTV-10-infected cells. Mock- and BTV-10-infected BSR-T7 cells were incubated in the absence (PMY-) or presence (PMY+) of puromycin (PMY) at 24 h pi. Cells were immunolabelled with antibodies against NS2 (green) and PMY (red); nuclei stained with DAPI (blue). The plot profiles display the fluorescence intensity values of PMY and NS2 measured along the white lines drawn in the merged images. Sizes of scale bars= 10 μm.

BTv-10

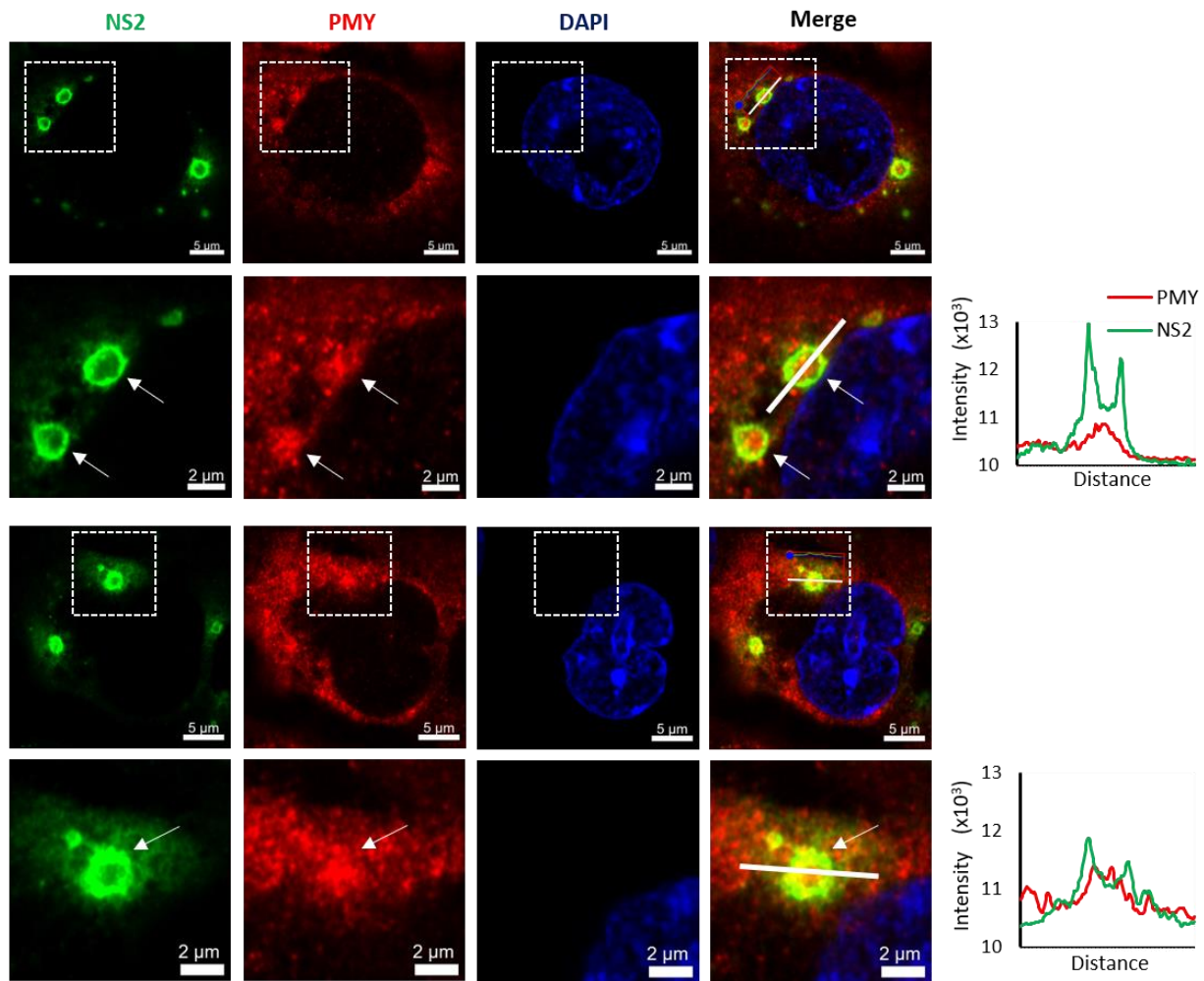


Fig. 2.19. Confocal microscopy images showing the colocalisation of BTv-10 VIBs with large foci of actively translating ribosomes (arrows). BTv-10-infected BSR-T7 cells were subjected to the RPM assay, fixed at 24 h pi and immunolabelled with antibodies against NS2 (green) and PMY (red); nuclei stained with DAPI (blue). Each second row shows a higher magnification view of the areas demarcated by the white block in the images above it. The plot profiles display the fluorescence intensity values of PMY and NS2 measured along the white lines drawn in the merged images. Sizes of scale bars as indicated.

The elevated detection of PMY in the VIBs provides evidence for a novel role for these virus replication factories in compartmentalising virus translation. As host translation occurs in the cytoplasm, and the VIBs contain an abundance of virus cores and RNAs (Brookes *et al.* 1993), the heightened intensity of PMY within the VIBs compared to disperse cytoplasmic intensity indicates that the VIBs might act as a hub for up-regulating virus translation above host levels, possibly by sequestering host protein synthesis machinery. The occurrence of this phenomenon in two different serotypes of AHSV and in the related BTv indicates that this is a broad functionality of orbivirus VIBs.

2.3.2.2. *In the absence of other viral proteins and RNAs, puromycin-incorporated polypeptides do not preferentially localise to the VIB-like structures*

VIBs formed during AHSV infection consist of an NS2 matrix, but also contain the five minor structural proteins of the virus and the 10 viral genome segments in ssRNA and dsRNA form (Huisman *et al.* 1987; Roy *et al.* 1994; Modrof *et al.* 2005; Lymperopoulos *et al.* 2006; Kar *et al.* 2007). Any or a combination of these components could be involved in mediating the redistribution of actively translating ribosomes to the VIBs. To investigate this, NS2 was transiently expressed in the mammalian cell line used for the AHSV infection. BSR-T7 cells were transfected with phCMV-NS2 to express NS2, or with phCMV-NS2-eGFP to express a fluorescing NS2-eGFP fusion protein that would not require NS2 labelling. Cells were subjected to the RPM assay at 48 h pt, when they were fixed, labelled for AHSV NS2 (in the case of phCMV-NS2) and PMY and analysed via CLSM. Anti-rabbit AlexaFluor 488 and anti-mouse AlexaFluor 594 secondary antibodies were used for detecting anti-NS2 and anti-PMY primary antibodies respectively. Mock-transfected (containing all transfection reagents except DNA) and plasmid-transfected cells which were pre-treated with emetine but PMY omitted (PMY-) were included as controls (Fig. 2.20 – 2.22).

Mock-, phCMV-NS2-and phCMV-NS2-eGFP-transfected PMY- cells showed negligible background, with a substantial increase in PMY signal in mock- and plasmid-transfected PMY+ cells (Fig. 2.20). In some of the PMY+ plasmid-transfected cells the PMY signal was not as strong as in PMY+ mock-transfected cells, which could possibly be due to cells being compromised as a result of foreign gene expression.

The distribution of PMY in mock- and plasmid-transfected cells was restricted to the cytoplasm. Similar to mock-transfected cells, PMY distributed diffusely in both phCMV-NS2- and phCMV-NS2-eGFP- transfected cells (Fig. 2.21 and 2.22). Where the VIB-like structures were localised, PMY was often present, however the intensity of PMY at these localisations was similar to that of the dispersedly distributed surrounding PMY signal. Plot profile intensity diagrams showed the intensity of PMY to remain relatively unchanged in the VIB-like foci when compared to the surrounding diffuse cytoplasmic intensities. As similar patterns were seen in phCMV-NS2- and phCMV-NS2-eGFP transfected cells, it confirmed that NS2 immunolabelling did not affect the appearance of the distribution pattern of PMY.

As no increased levels of PMY were present in VIB-like structures formed by either phCMV-NS2 or phCMV-NS2-eGFP, it can be hypothesised that translation is not up-regulated in these structures. With virus RNAs and proteins being absent in both these systems, it is expected that translation would not actively occur within the VIB-like structures when only NS2 is expressed. A reasonable hypothesis to consider is therefore that some active translating ribosomes simply get trapped as the NS2 aggregates form around it, but that protein synthesis is not promoted in VIB-like structures when NS2 is expressed individually. This confirms the requirement for viral RNAs and/or proteins other than NS2 to be present for active protein synthesis to occur

within the VIBs, and shows the stringency of the RPM assay to specifically detect a biological process in which all the molecules required for its execution are present.

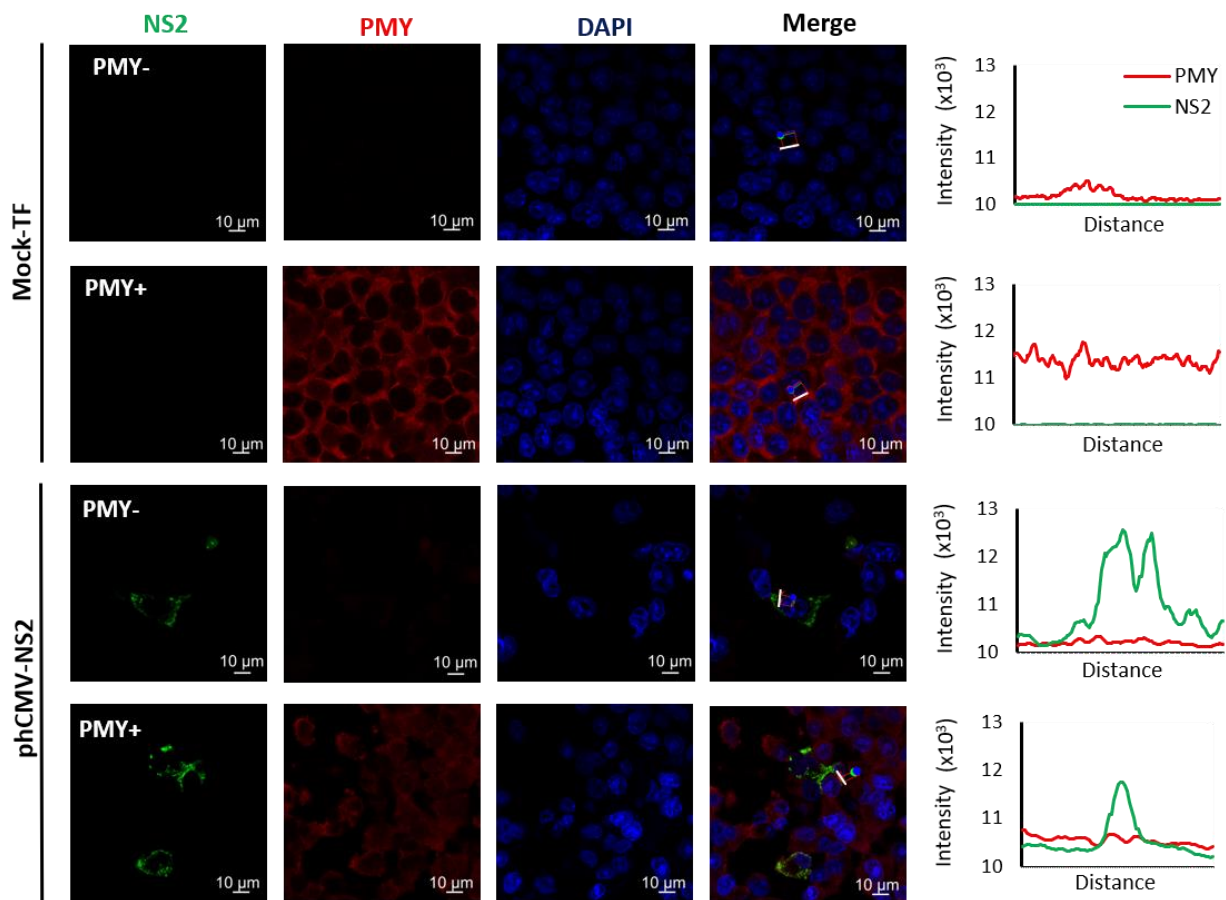


Fig. 2.20. Confocal microscopy images showing the distribution of actively translating ribosomes in mock- and phCMV-NS2-transfected cells. Mock- and phCMV-NS2-transfected BSR-T7 cells were incubated in the absence (PMY-) or presence (PMY+) of puromycin (PMY) at 48 h pt. Cells were immunolabelled with antibodies against NS2 (green) and PMY (red); nuclei stained with DAPI (blue). The plot profiles display the fluorescence intensity values of PMY and NS2 measured along the white lines drawn in the merged images. Sizes of scale bars as indicated.

phCMV-NS2

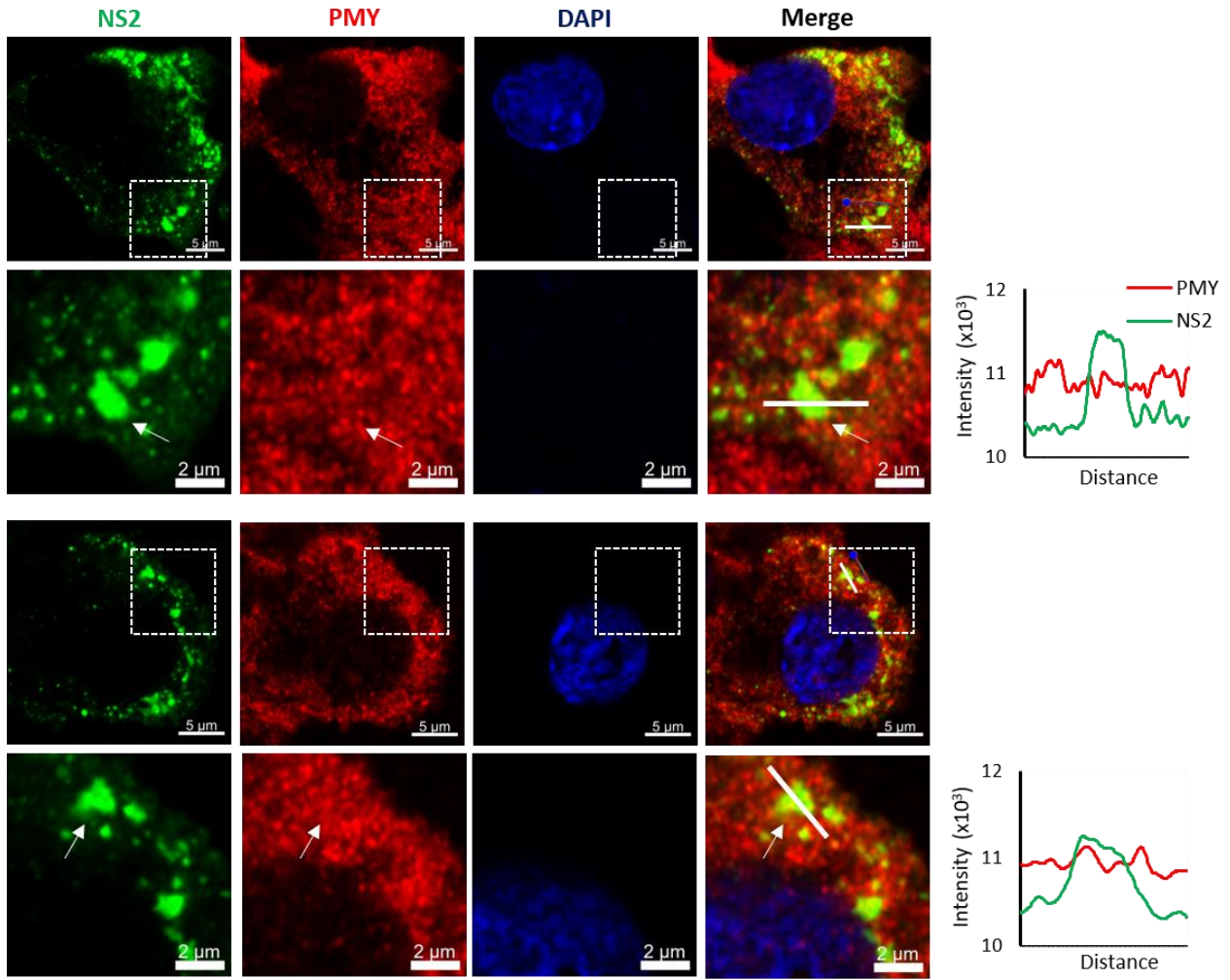


Fig. 2.21. Confocal microscopy images showing the even distribution of actively translating ribosomes in the cytoplasm and the VIB-like structures when NS2 is individually expressed. BSR-T7 cells were transfected with phCMV-NS2, subjected to the RPM assay at 48 h pt and fixed. Cells were dual immunolabelled for NS2 (green) and PMY (red); nuclei stained with DAPI (blue). Each second row shows a higher magnification view of the demarcated areas in the images above it. The plot profiles display the fluorescence intensity values of PMY and NS2 measured along the white lines drawn in the merged images. Sizes of scale bars as indicated.

phCMV-NS2-GFP

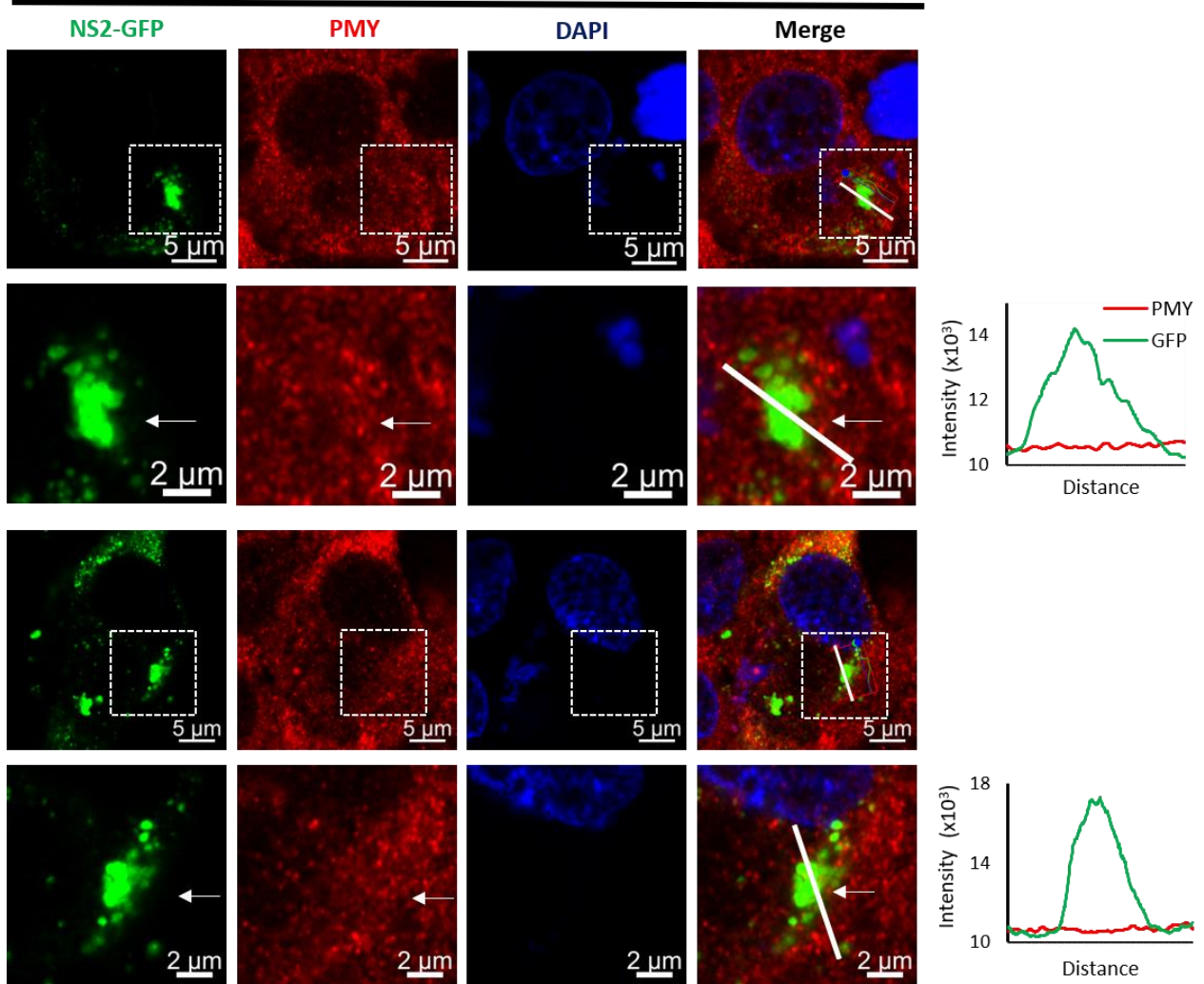


Fig. 2.22. Confocal microscopy images showing the unchanged distribution of actively protein synthesising ribosomes in the cytoplasm and the VIB-like structures when NS2 is individually expressed and not immunolabelled. BSR-T7 cells were transfected with phCMV-NS2-eGFP, subjected to the RPM assay and fixed at 48 h pt. Cells immunolabelled for PMY (red); nuclei stained with DAPI (blue). Each second row shows a higher magnification view of the demarcated areas in the images above it. The plot profiles display the fluorescence intensity values of PMY and NS2 measured along the white lines drawn in the merged images. Sizes of scale bars as indicated.

2.3.3. Determining the sub-structural localisation profiles of different host translation factors relative to the VIBs

The detection of active protein synthesis within the VIBs provided new questions as to how components of the protein synthesis apparatus are arranged to orchestrate translation within the VIBs. Previously, different components of the eukaryotic translation initiation complex were shown to localise to variable regions of reovirus viral factories (Desmet *et al.* 2014). In this section, the distribution profiles of four different components involved in eukaryotic translation were characterised. The aim was to investigate whether these cellular factors could be detected within AHSV VIBs, and if so to determine whether they displayed any specific sub-structural localisation. The components tested were L11 and S3, proteins of the large 60S and small 40S ribosomal subunits respectively, and eIF3 θ and eIF4E, eukaryotic translation initiation factors.

2.3.3.1. Ribosomal subunit proteins L11 and S3 and translation initiation factors eIF3 θ and eIF4E show different sub-structural localisations relative to AHSV-5 VIBs

Before commencing with immunofluorescence analyses, the expression of L11, S3, eIF3 θ and eIF4E in BSR-T7 cells and the specificity of their detection with available antibodies were tested.

BSR-T7 cell lysates were subjected to Western blotting for L11, S3, eIF4E or eIF3 θ (Fig. 2.24). Cells subjected to immunoblotting for L11 did not show band formation (data not shown), and is suspected to be attributed to the unsuitability of the anti-L11 antibody for Western blot applications, as per antibody manufacturer's specifications. Immunoblots of cells blotted for S3 showed a distinct band of expected size, 33 kDa (Fig. 2.24 A). Cells blotted for eIF3 θ (Fig. 2.24 B) or eIF4E (Fig. 2.24 C) showed a distinct band of 170 kDa or 28 kDa respectively, with minimal background. In each case, background was below-detectable in BSR-T7 cells which thus confirms the specificity of the available anti-S3, anti-eIF3 θ and anti-eIF4E antibodies to their respective antigens.

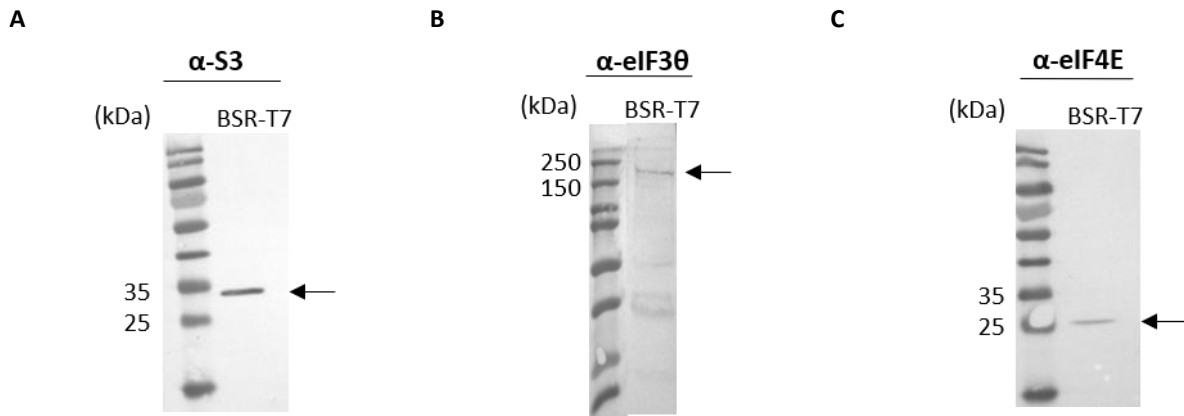


Fig. 2.24 Western blot images showing the expression of a small ribosomal subunit protein and two eukaryotic translation initiation factors and the specificity of their detection with available antibodies. BSR-T7 whole cell lysates were separated by SDS-PAGE and subjected to immunoblotting for (A) S3, (B) eIF3 θ or (C) eIF4E. Arrows show to the specific bands displayed on each immunoblot. α = anti. Sizes of the protein marker as indicated to the left.

Next, the distribution profiles of L11, S3, eIF3 θ and eIF4E and their localisation relative to the VIBs were characterised (Fig. 2.25 – 2.28). BSR-T7 cells were infected with AHSV-4 or AHSV-5 and fixed at 24 h pi. Cells were subjected to dual immunolabelling for AHSV NS2 (with anti-rabbit AlexaFluor 488 as secondary antibody) and either L11, S3, eIF3 θ or eIF4E (with anti-mouse AlexaFluor 594 as secondary antibody), and analysed via CLSM. To determine whether the sequence in which the antibodies were added affected the localisation pattern of the fluorophores, dual labelling was done sequentially by adding either the anti-NS2 or the anti-S3, eIF3 θ or eIF4E first, and then the alternative antibody at a later stage. The images obtained from the two labelling protocols were then compared.

In the first set of experiments we labelled for L11, a protein component of the large ribosomal subunit (Fig. 2.25). Mock-infected cells showed a diffuse distribution of L11 in the cytoplasm and the nucleus, which was as predicted based on the antibody information sheet provided by the supplier. In AHSV-4- infected cells, L11 shifted from having only a diffuse distribution to also forming large foci similar in size and morphology to the VIBs. Dual labelling confirmed that these large L11 foci colocalised with the VIBs, where they showed a brighter intensity compared to their dispersedly distributed counterparts. Intensity plot profiles showed an elevation in L11 intensity towards the middle of the VIBs. This indicates that L11 is present throughout the VIBs, especially at the central regions. This could be indicative of sequestration of L11 (and probably ribosomes) from host utilisation in the cytoplasm to be preferentially utilised for virus translation within the VIBs.

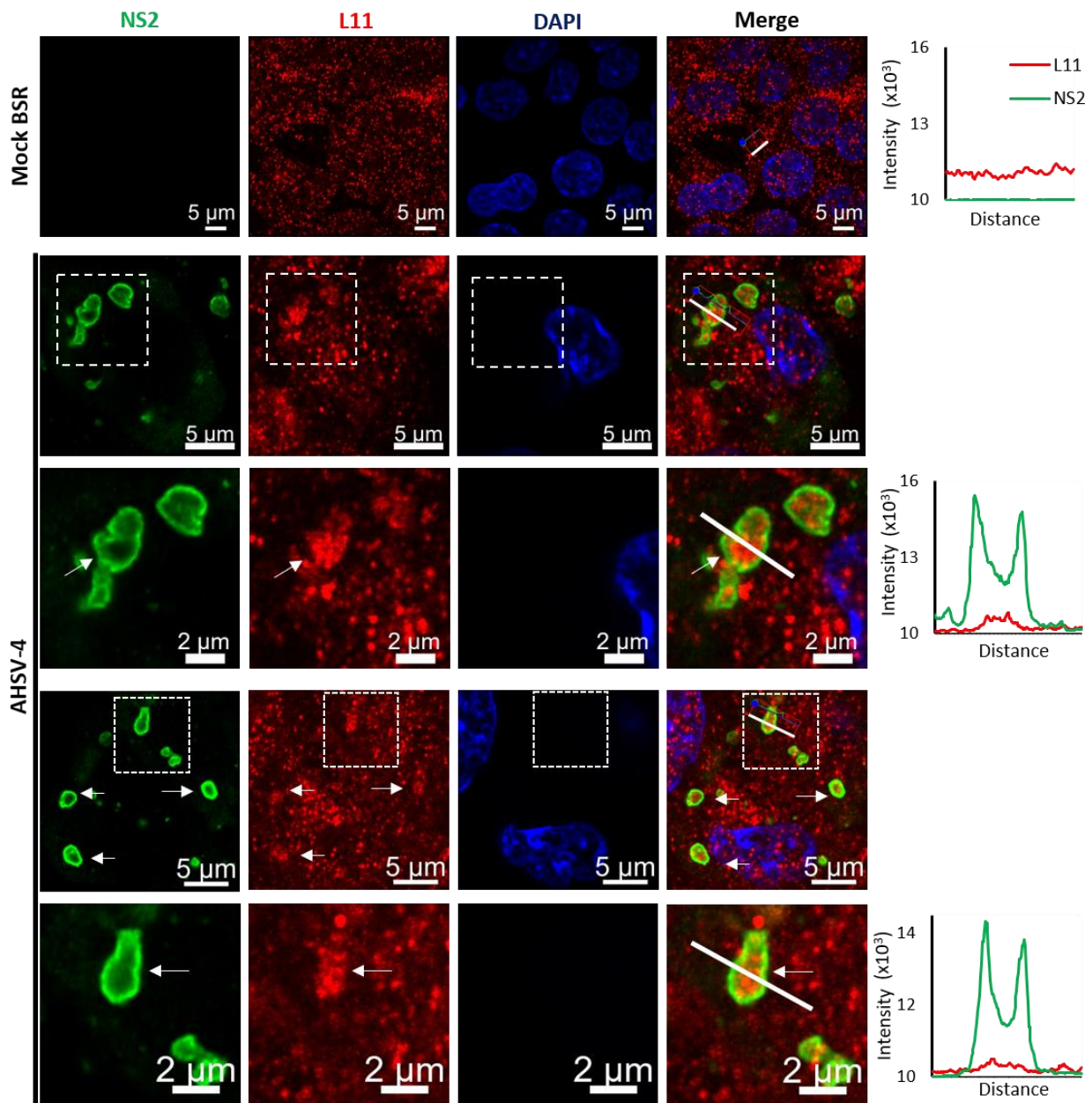


Fig. 2.25. Confocal microscopy images showing the colocalisation of L11 foci with the VIBs (arrows). Mock and AHSV-4-infected BSR-T7 cells were fixed at 24 h pi and dual immunolabelled for NS2 (green) and L11 (red); nuclei stained with DAPI (blue). Each second row of the AHSV-4 images shows a higher magnification view of the demarcated areas in the images above. The plot profiles display the fluorescence intensity values of L11 and NS2 measured along the white lines drawn in the merged images. Sizes of scale bars as indicated.

Next, we tested for the S3 protein of the small ribosomal subunit (Fig. 2.26). S3 showed a diffuse distribution that was restricted to the cytoplasm, which was slightly different from what was predicted by the antibody information sheet, which also showed a nuclear distribution. The reason for this difference in localisation pattern might be due to the different cell type and fixation method used by the manufacturer. Interestingly, both mock- and AHSV-5-infected cells showed distinct cytoplasmic areas that were devoid of S3, especially at the perinuclear regions. In AHSV-5-infected cells, S3 showed gaps in signal where the VIBs were localised.

At first glance, S3 appeared to be completely absent from the VIBs. However, intensity plot profiles revealed a concave shape of S3 signal within the VIBs, which declined at the centre and increased towards the margins of the VIBs. The intensity of S3 at the margins of the VIBs was either heightened or relatively similar to the dispersed cytoplasmic intensity. The sequence of immunolabelling did not affect the trends of intensity profiles of either NS2 or S3, but only resulted in an overall increase in S3 intensity due to longer incubation time with anti-S3 when anti-NS2 was added first.

S3 is therefore not completely excluded from the VIBs. Rather, its localisation is restricted to a distinct sub-structural peripheral localisation within the VIBs. This phenomenon can be either biological in nature, indicating the active exclusion of S3 from the central regions for its selective use at the margins, or due to technical factors relating to the degree of anti-S3 penetration efficiency within the dense matrix of the VIBs.

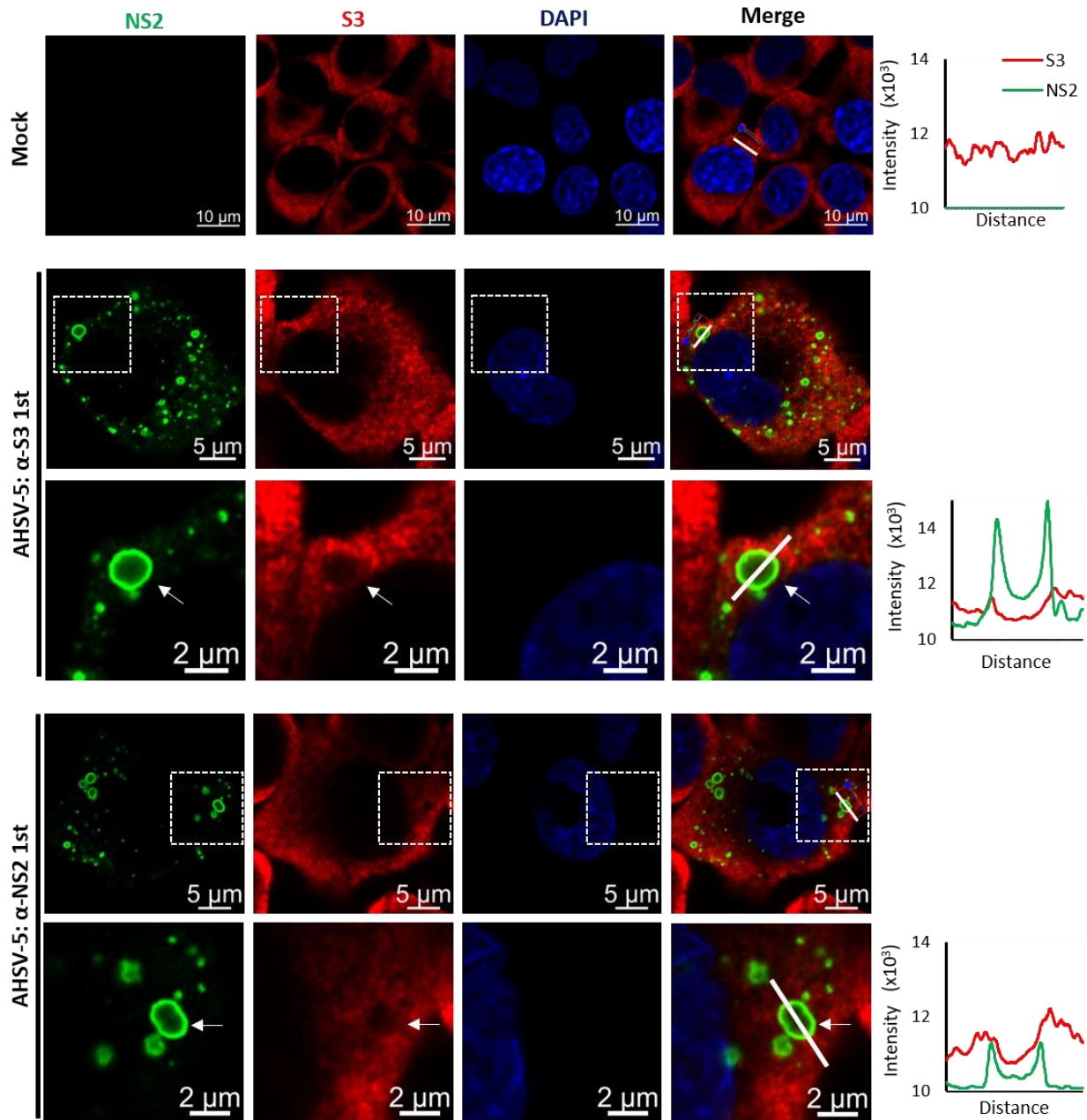


Fig. 2.26. Confocal microscopy images showing the localisation of S3 to the margins of the VIBs (arrows). BSR-T7 cells were infected with AHSV-5 and fixed at 24 h pi, mock-infected cells used as control. Cells were dual immunolabelled for NS2 (green) and S3 (red); nuclei stained with DAPI (blue). Each second row of the AHSV-5 images shows a higher magnification view of the demarcated areas in the images above. The plot profiles display the fluorescence intensity values of S3 and NS2 measured along the white lines drawn in the merged images. α = anti. Sizes of scale bars as indicated.

Lastly, we investigated the localisation of two of the eukaryotic translation initiation factors, namely eIF30 and eIF4E. Mock-infected cells labelled for eIF30 showed a diffuse distribution pattern localised to the nucleus and the cytoplasm (Fig. 2.27), differing from the manufacturer's description of strictly cytoplasmic and membrane localisation. In AHSV-5-infected cells, eIF30 distributed both diffusely and formed bright large foci resembling the VIBs. These foci of eIF30 localised throughout the VIBs, and showed a brighter intensity

compared to the diffusely spread eIF3θ in the cytoplasm. Plot profile intensity diagrams showed an increase in eIF3θ signal intensity at the central regions of the VIBs that exceeded that of the adjacent cytoplasmic intensity. While the order of immunolabelling steps could increase the overall intensity of eIF3θ, the trends of the intensity profiles of eIF3θ relative to NS2 were not affected. In each case, eIF3θ peaked in intensity at the central regions of the VIBs.

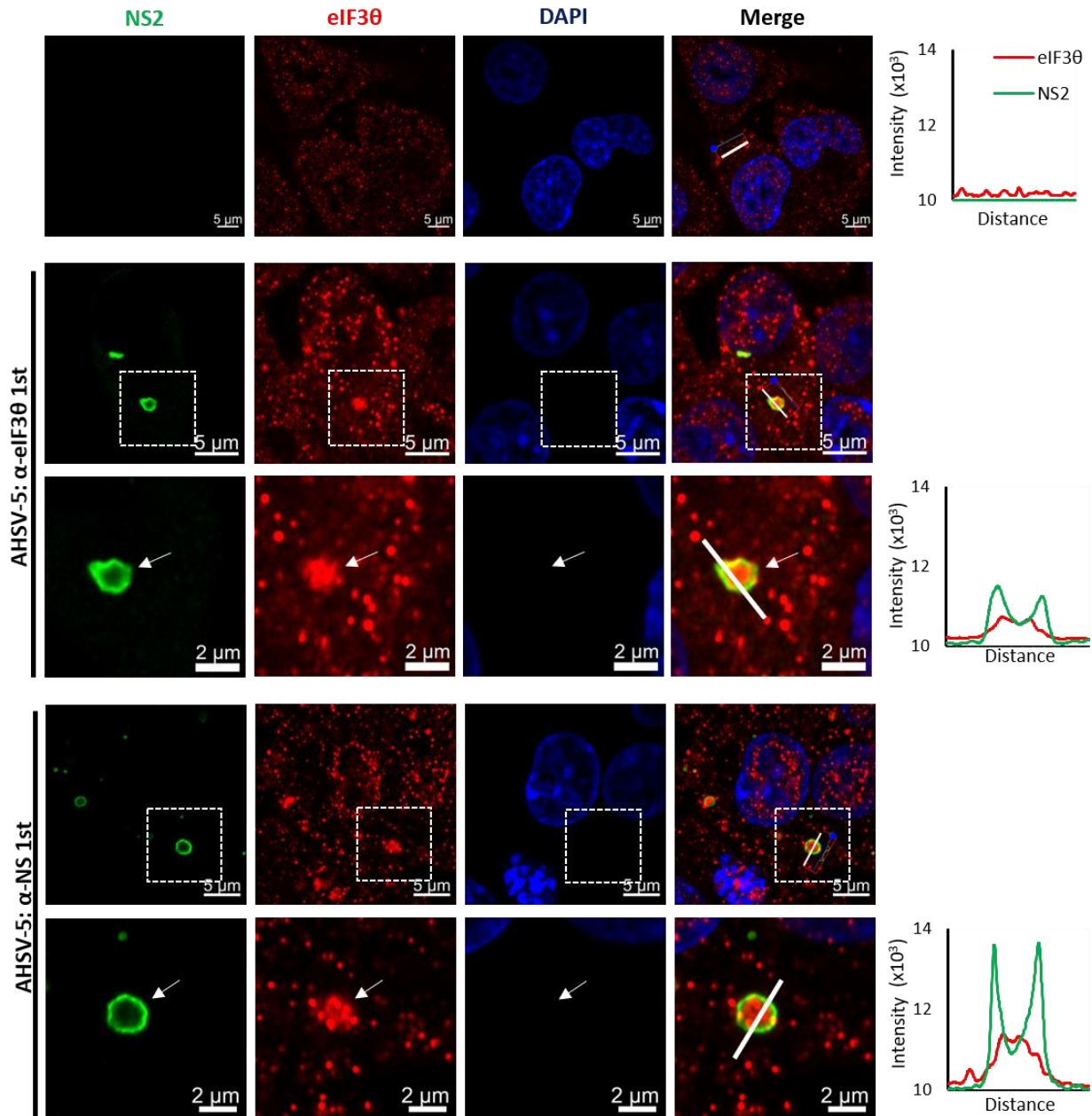


Fig. 2.27. Confocal microscopy images showing the localisation of eIF3θ foci to the VIBs (arrows). BSR-T7 cells were mock-infected or infected with AHSV-5 and fixed at 24 h pi. Cells were subjected to dual immunolabelling for NS2 (green) and eIF3θ (red); nuclei stained with DAPI (blue). Each second row in the AHSV-5 images shows a higher magnification view of the demarcated areas in the images above. The plot profiles display the fluorescence intensity values of eIF3θ and NS2 measured along the white lines drawn in the merged images. α= anti. Sizes of scale bars as indicated.

Mock- and AHSV-5-infected cells labelled for eIF4E showed a dispersed cytoplasmic distribution, with fainter labelling in the nucleus (Fig. 2.28), similar to what is described by the manufacturer. In AHSV-5-infected cells, where the VIBs were localised, eIF4E showed a decreased intensity. Plot profiles revealed a dip in intensity of eIF4E towards the central regions of the VIBs, which increased towards the margins. In some cases, the intensity of eIF4E at the margins was similar and in other cases increased compared to the dispersed cytoplasmic intensity. The trends in intensities of eIF4E relative to NS2 were not affected by immunolabelling sequence. The selective presence of eIF4E at the margins of the VIBs might either be indicative of a specialised localisation-specific role of eIF4E or due to inefficient penetration of anti-eIF4E.

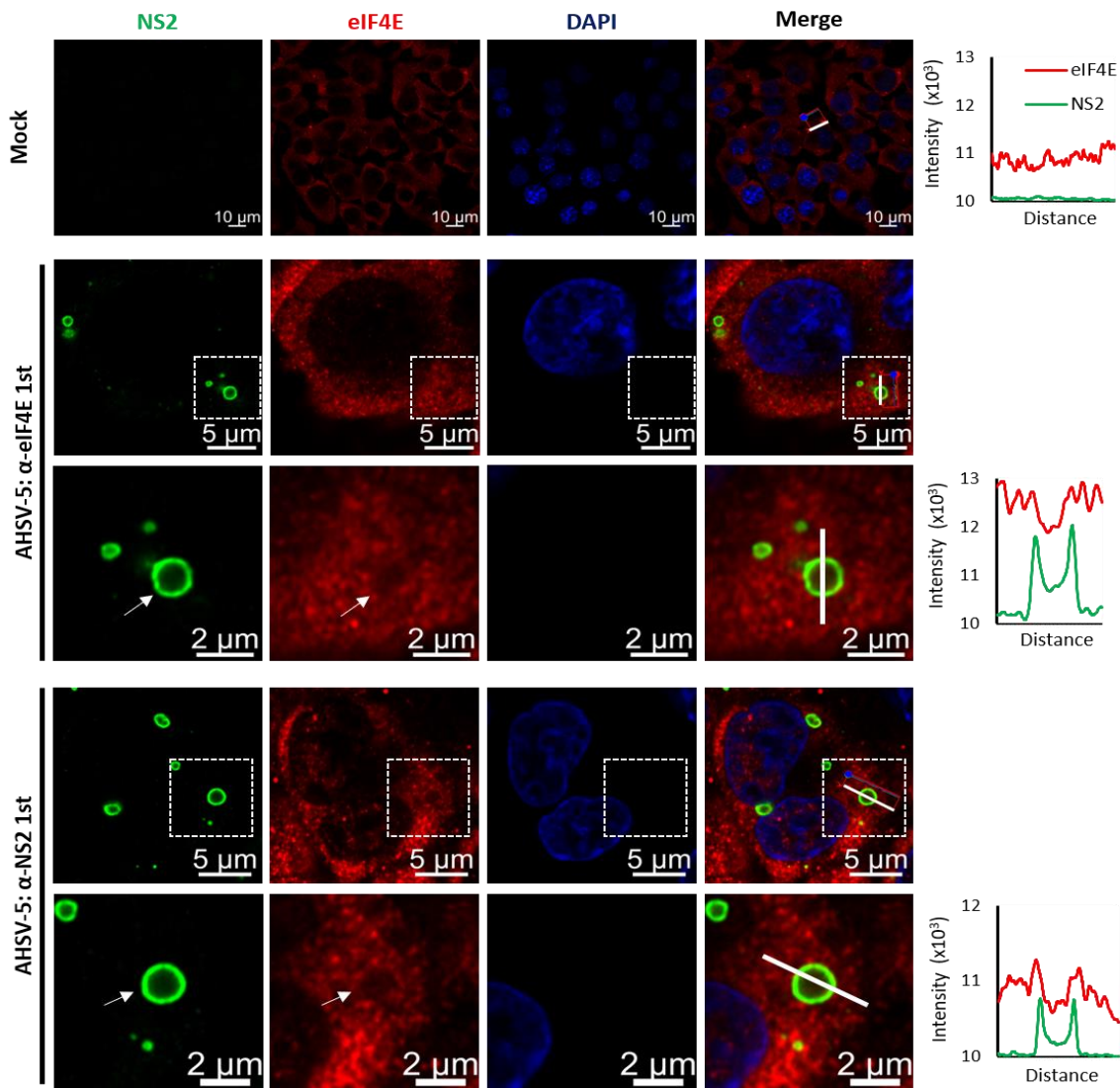


Fig. 2.28. Confocal microscopy images showing the localisation of eIF4E to the margins of the VIBs (arrows). BSR-T7 cells were infected with AHSV-5 and fixed at 24 h pi. Cells were subjected to dual immunolabelling for NS2 (green) and eIF4E (red); nuclei stained with DAPI (blue). Each second row in the AHSV-5 images shows a higher magnification view of the demarcated areas in the images above. The plot profiles display the fluorescence intensity values of eIF4E and NS2 measured along the white lines drawn in the merged images. α = anti. Sizes of scale bars as indicated.

2.3.3.2. *The interaction of the VIBs and the host translation factors is mediated solely by NS2*

The VIBs of orbiviruses are complex structures containing many different virus structural and non-structural proteins, nucleic acids and particles (Brookes *et al.* 1993). In this section, it was investigated whether other VIB components than NS2 are required to mediate the interaction with host cell translational machinery. For this we used both the recombinant baculovirus BacNS2 and the plasmid pHCMV-NS2 to recombinantly express NS2 in insect and mammalian cells respectively outside the context of an AHSV infection.

Sf9 cells were infected with BacNS2, and BSR-T7 cells transfected with pHCMV-NS2, to express NS2 and form VIB-like structures. At 48 h pi, Sf9 cells were fixed with PFA or methanol-acetone, and at 48 h pt, BSR-T7 cells were fixed and subjected to dual immunolabelling for NS2 (with anti-rabbit AlexaFluor 488 as secondary antibody) and either L11, S3, eIF3 θ or eIF4E (with anti-mouse AlexaFluor 594 as secondary antibody) (Fig. 2.29 – 2.33). Immunolabelling was done sequentially, and the sequences in which the antibodies were added were tested for their effects on the localisation profiles of the antigens.

Sf9 cells fixed with methanol-acetone displayed below detectable L11 fluorescence (data not shown), and were thus not used for further analyses. In mock-infected Sf9 cells fixed with PFA, L11 displayed a diffuse nuclear and cytoplasmic distribution, with small punctate foci forming in some cells (Fig. 2.29). In BacNS2-infected Sf9 cells, L11 often formed large bright structures that resembled VIB-like structures typically induced by an infection with BacNS2. These L11 foci colocalised with the VIB-like structures. Plot profiles showed a peak in intensity of L11 within the VIB-like structures above adjacent cytoplasmic levels. BSR-T7 cells transfected with pHCMV-NS2 showed a similar profile, with bright L11 foci strongly colocalising with VIB-like structures (Fig. 2.30).

Sf9 cells fixed with either methanol acetone or PFA showed to be unsuitable for immunolabelling the antigens of S3, eIF3 θ and eIF4E, as the intensities of the antigens were substantially lower compared to what is typically seen in BSR-T7 cells (data not shown). This might be due to antibodies being specific to antigens of mammalian origin. For this reason, analyses were restricted to BSR-T7 cells transfected with pHCMV-NS2.

S3 in mock- and pHCMV-NS2-transfected BSR-T7 cells showed a dispersed cytoplasmic distribution, with some cellular compartments being devoid of S3 signal (Fig. 2.31). The VIB-like structures did not colocalise with S3, but rather showed a decrease in signal towards the centres. Plot profiles showed an upward curve of S3 towards the margins of the VIB-like structures.

The distribution of eIF3 θ in mock-transfected and pHCMV-NS2-transfected BSR-T7 cells showed a diffuse nuclear and cytoplasmic profile (Fig. 2.32). In pHCMV-NS2 transfected cells, eIF3 θ also distributed as foci that were similar in size and morphology to VIB-like structures. These foci of eIF3 θ colocalised with the VIB-like structures induced by pHCMV-NS2. The central regions of the VIB-like structures showed a high signal intensity of eIF3 θ that exceeded that of dispersedly distributed eIF3 θ in the cytoplasm.

A homogenous cytoplasmic distribution of eIF4E along with the formation of small punctate foci was shown in mock- and phCMV-NS2-transfected cells (Fig. 2.33). At the localisation of the VIB-like structures in phCMV-NS2-transfected cells, eIF4E showed a decrease in intensity. Plot profiles showed an increase in eIF4E intensity towards the margins of the VIB-like structures.

None of the profile trends were affected by the sequence in which immunolabelling was conducted.

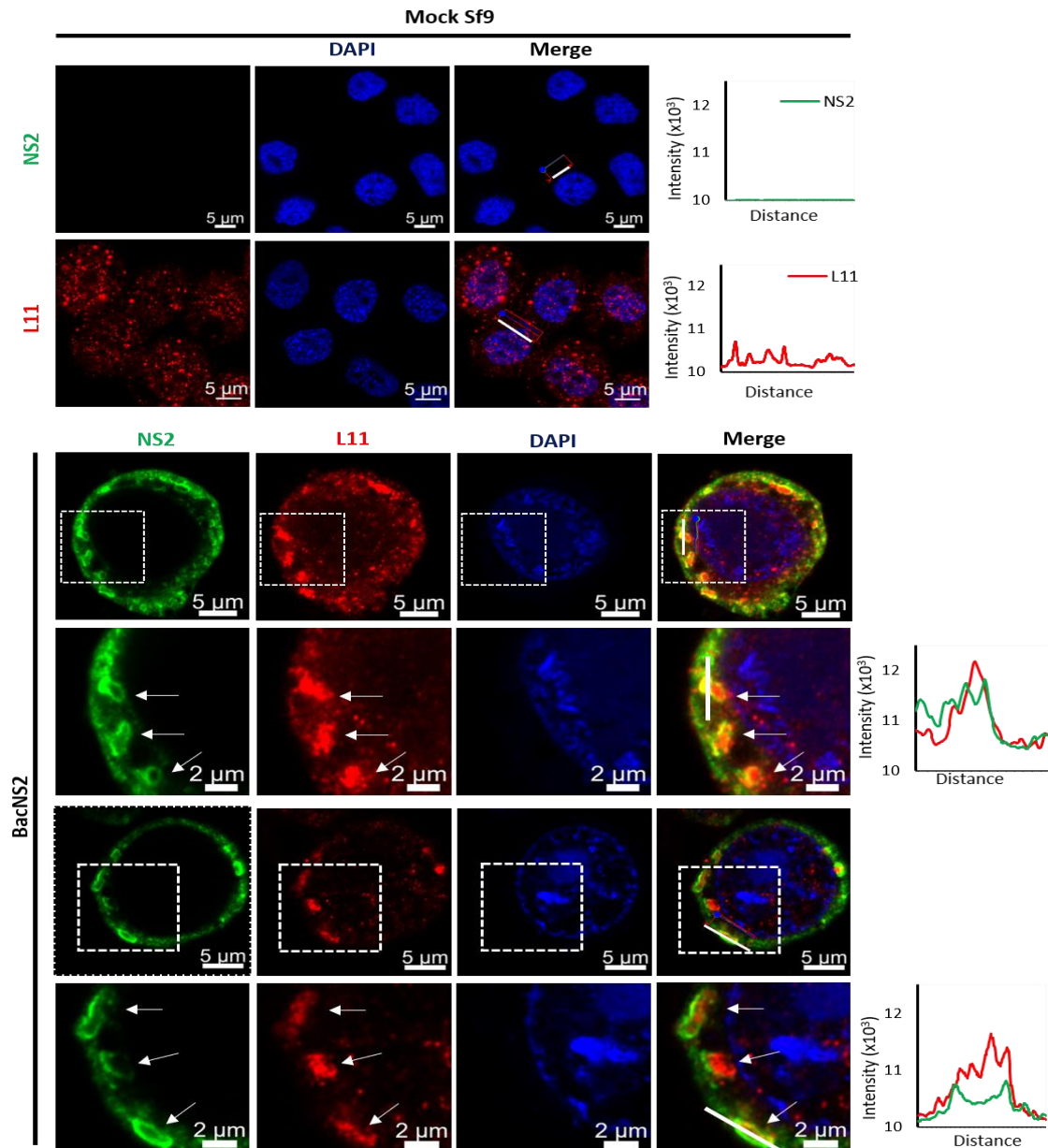


Fig. 2.29. Confocal microscopy images showing the colocalisation of L11 with VIB-like foci (arrows) when NS2 is expressed from a recombinant baculovirus. Sf9 cells were infected with BacNS2 and fixed at 48 h pi. Cells were immunolabelled for NS2 (green) and L11 (red); nuclei stained with DAPI (blue). Each second row in the BacNS2 images shows a higher magnification view of the demarcated areas in the images above. The plot profiles display the fluorescence intensity values of L11 and NS2 measured along the white lines drawn in the merged images. Sizes of scale bars as indicated.

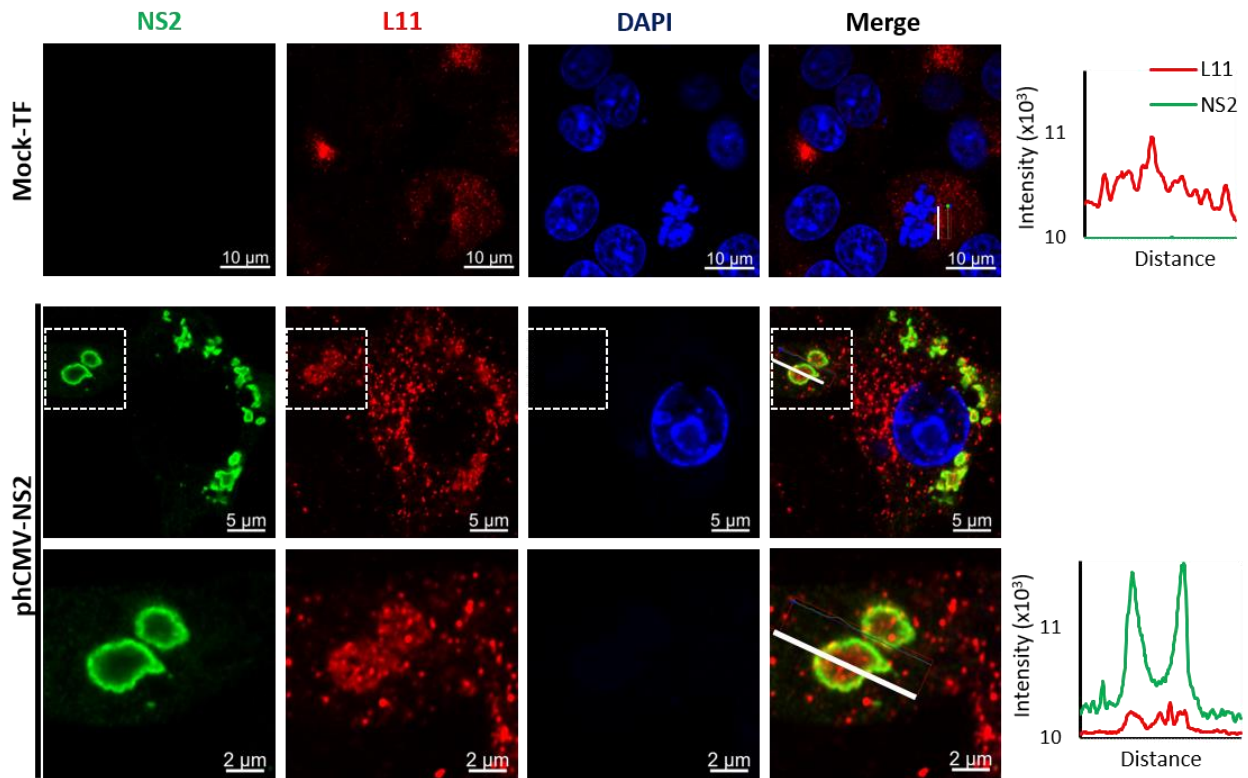


Fig. 2.30. Confocal microscopy images showing the colocalisation of L11 with VIB-like foci (arrows) when NS2 is expressed from a plasmid. BSR-T7 cells were transfected with phCMV-NS2 and fixed at 48 h pt. Cells were immunolabelled for NS2 (green) and L11 (red); nuclei stained with DAPI (blue). The second row in the phCMV-NS2 images shows a higher magnification view of the demarcated areas in the images above. The plot profiles display the fluorescence intensity values of L11 and NS2 measured along the white lines drawn in the merged images. Mock-TF= mock-transfected. Sizes of scale bars as indicated.

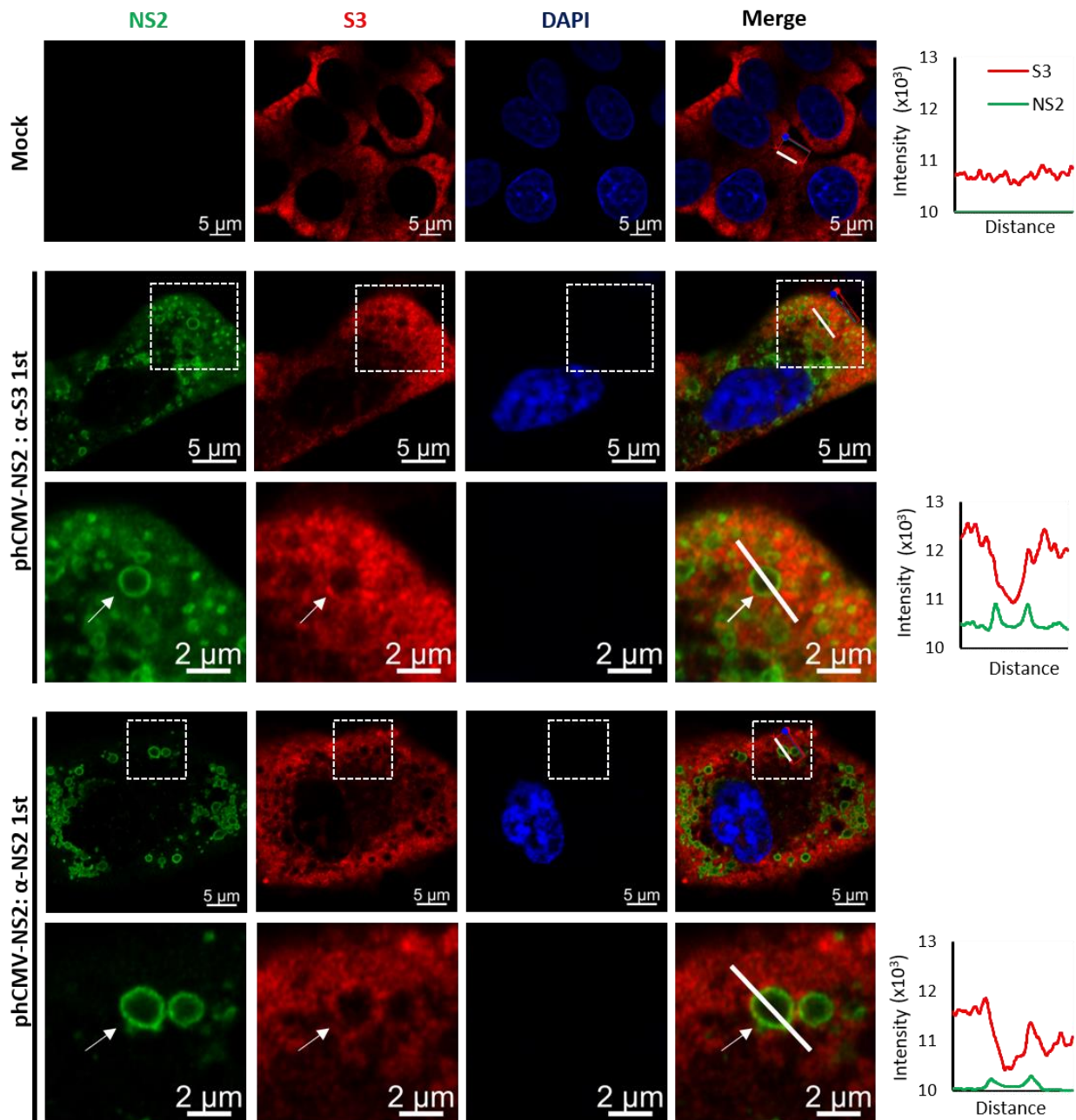


Fig. 2.31. Confocal microscopy images showing the localisation of S3 to the margins of the VIBs-like structures (arrows) when NS2 is expressed from a plasmid. BSR-T7 cells were transfected with phCMV-NS2 and fixed at 48 h post-transfection. Cells were dual immunolabelled for NS2 (green) and S3 (red); nuclei stained with DAPI (blue). Each second row in the phCMV-NS2 images shows a higher magnification view of the demarcated areas in the images above. The plot profiles display the fluorescence intensity values of S3 and NS2 measured along the white lines drawn in the merged images. α= anti. Sizes of scale bars as indicated.

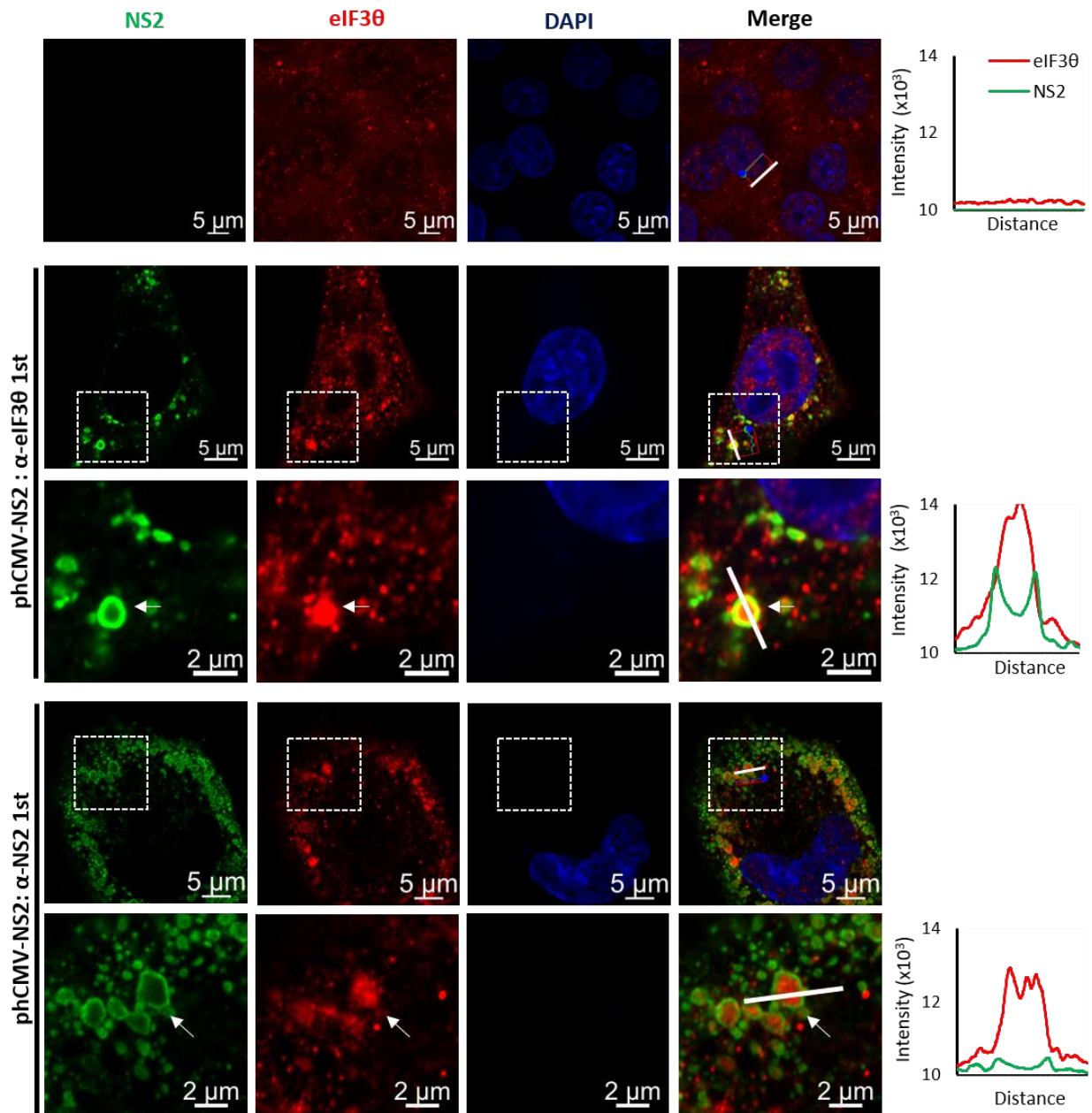


Fig. 2.32. Confocal microscopy images showing the localisation of eIF30 to the VIB-like structures (arrows) when NS2 is expressed from a plasmid. BSR-T7 cells were transfected with phCMV-NS2 and fixed at 48 h pt. Cells were dual immunolabelled for NS2 (green) and eIF30 (red); nuclei stained with DAPI (blue). Each second row in the phCMV-NS2 images shows a higher magnification view of the demarcated areas in the images above. The plot profiles display the fluorescence intensity values of eIF30 and NS2 measured along the white lines drawn in the merged images. α= anti. Sizes of scale bars as indicated.

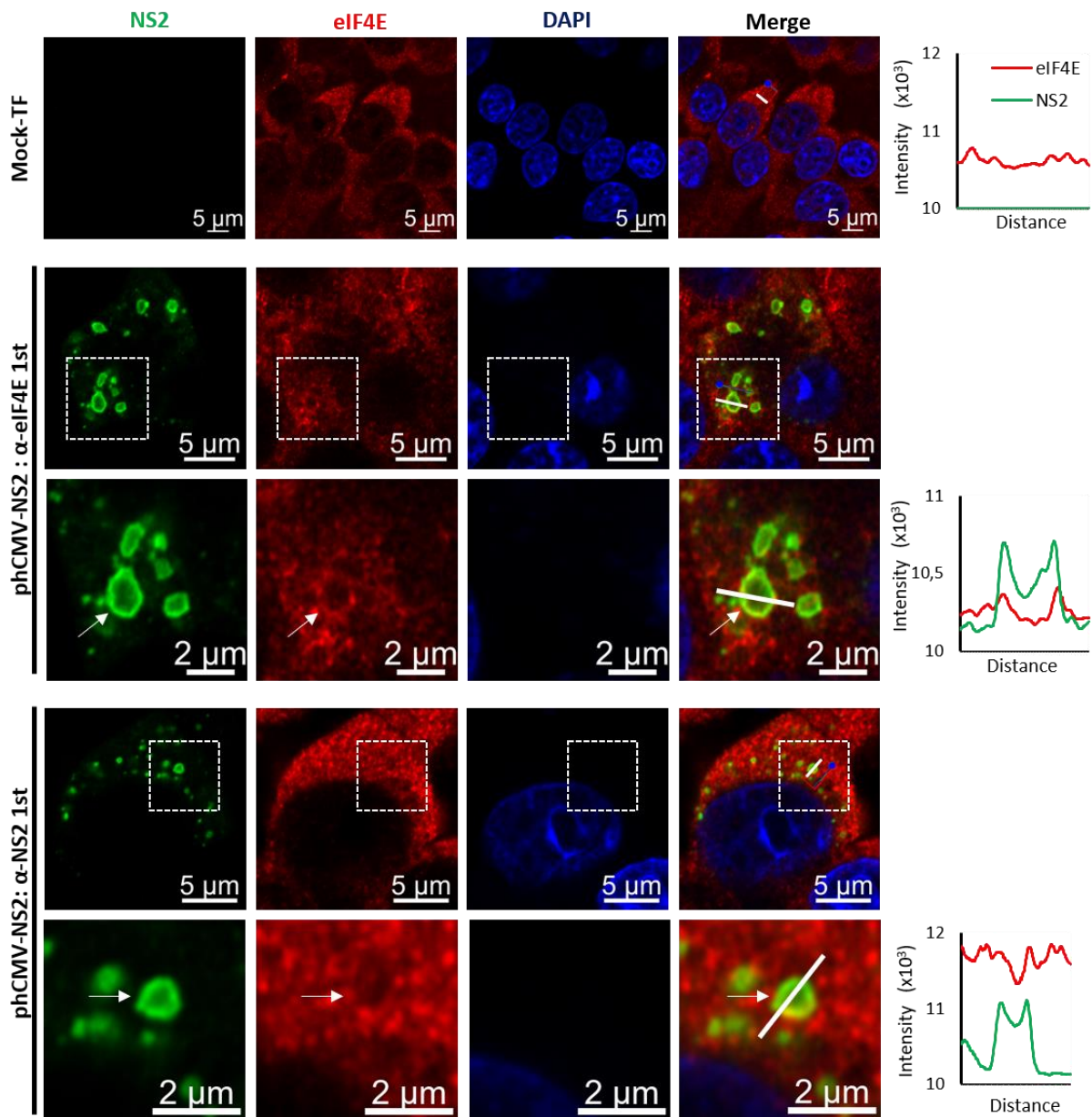


Fig. 2.33. Confocal microscopy images showing the localisation of eIF4E to the margins of the VIB-like structures when NS2 is expressed from a plasmid. BSR-T7 cells were transfected with phCMV-NS2 and fixed at 48 h pt. Cells were immunolabelled for NS2 (green) and eIF4E (red); nuclei stained with DAPI (blue). Each second row in the phCMV-NS2 images shows a higher magnification view of the demarcated areas in the images above. The plot profiles display the fluorescence intensity values of eIF4E and NS2 measured along the white lines drawn in the merged images. α= anti. Sizes of scale bars as indicated.

In each case tested, the localisation profile of L11, S3, eIF3θ and eIF4E relative to the VIB-like structures in an NS2 expression system was similar to that seen in the context of an AHSV infection. This indicates that the presence of only NS2 in the VIBs is sufficient to mediate the interaction of the VIBs with these components of the protein synthesis machinery.

2.3.3.3. *NS2 protein complexes were detected via immunoblotting*

Co-immunoprecipitation (co-IP) studies provide useful tools for detecting protein-protein interactions. As confocal microscopy analyses revealed colocalisation between the VIBs and specific components of the host translation apparatus when NS2 was expressed recombinantly, it was attempted to verify their interactions by co-IP.

First, the efficacy of the anti-NS2 serum and a pulldown system using protein A-conjugated magnetic beads to purify NS2 from AHSV-infected cell lysates was established. BSR-T7 cells were infected with AHSV-5, and harvested at 24 h pi; mock-infected cell lysates were used as controls. NS2 was immunoprecipitated (IP) from whole cell lysates using anti-AHSV NS2 serum and protein A magnetic beads. The purified NS2 samples were subjected to Western blotting for AHSV NS2, using non-IP whole-cell lysates (W1 and W2) as controls. Western blots revealed successful purification of NS2 from AHSV-5 cell lysates, that were absent from the mock-infected controls (Fig. 2.34 A). Interestingly, the single lower molecular weight non-specific bands often detected in whole cell lysates, as seen in W1 and W2, were not detected in the immunoprecipitated NS2 samples. It is suspected that the degraded fragments of NS2 do not bind effectively to the antibodies in the protein A bead system.

Successful preservation of protein-protein interactions is dependent upon a cross-linking step to maintain native protein complexes. BSR-T7 cells were infected with AHSV-5 and whole cell lysates were subjected to cross-linking with 0,8% PFA, followed by quenching with glycine. Mock-infected and infected cells treated with the same conditions without PFA crosslinking were included as controls. The samples were subjected to Western blotting for NS2 to detect mobility shift, indicative of NS2 interacting with other proteins. Cross-linked whole cell lysates showed higher molecular weight bands for NS2, between 100 and 170 kDa, along with the 42 kDa size band representing non-interacting NS2 (Fig. 2.34 B). Non-cross-linked AHSV-infected cells showed NS2 of only 42 kDa in size, and mock-infected cells were devoid of NS2. This shows that cross-linking with 0,8% PFA successfully preserved at least some of the native protein complexes of NS2 interactions.

It was next tested whether NS2 protein interactions resulting from PFA cross-linking would remain intact through the IP process. BSR-T7 cells were infected with AHSV-5 and harvested at 24 h pi. Cell lysates were cross-linked with 0,8% PFA, quenched with glycine and subjected to IP for NS2 using protein A magnetic beads. Mock- and virus-infected whole cell lysates and non-cross linked NS2 purified samples were included as controls. Samples were subjected to Western blotting for NS2. All AHSV-5-infected cells showed a band of 42 kDa in size representing NS2, that was absent in all mock-infected cells (Fig. 2.34 C). Cross-linked whole cell lysates and NS2 purified samples from AHSV-5-infected cells showed higher molecular weight bands between 80 and 130 and above 170 kDa. These larger bands were absent in non-cross-linked AHSV-5-infected samples. The cross-linked NS2 purified samples showed a higher intensity compared to cross-linked whole

cell lysates. This confirms that 0.8% PFA cross-linking maintained NS2 protein complexes through the IP process.

It was next attempted to determine whether the NS2 bands showing mobility shift tested positive for interactions with eIF3θ and/or eIF4E. BSR-T7 cells were infected with AHSV-5 and lysed at 24 h pi. Cell lysates were cross-linked with 0,8% PFA and subjected to NS2 IP with protein A magnetic beads. Samples were then subjected to Western blotting for eIF3θ or eIF4E. Western blots did not reveal any bands for these translation factors on the blots (data not shown).

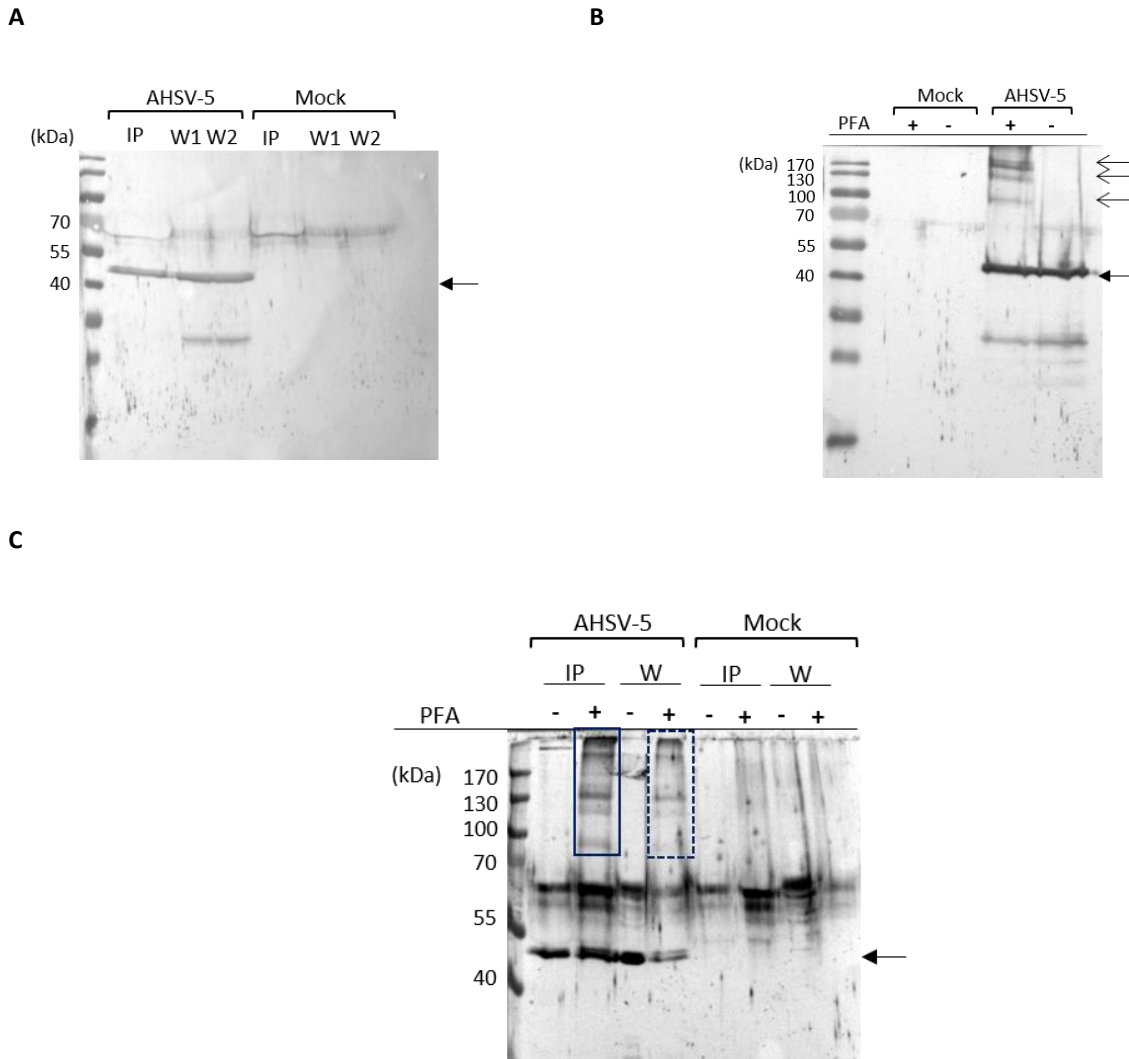


Fig. 2.34. Western blot images for NS2 showing successful immunoprecipitation; cross-linking and immunoprecipitation of cross-linked NS2 complexes. BSR-T7 cells were infected with AHSV-5 and harvested at 24 h pi. Cells were (A) subjected to IP for AHSV NS2, (B) subjected to cross-linking with 0,8% PFA, and (C) subjected to cross-linking and IP for AHSV NS2. Cell lysates were separated by SDS-PAGE and immunoblotted for NS2. (W)= whole-cell lysates of different volumes; (+)= with PFA cross-linking; (-)= without PFA cross-linking. Open arrowheads show NS2 that underwent mobility shift; solid arrowheads show 42 kDa monomeric NS2; dashed and solid boxes show the intensities of the high molecular weight NS2-containing complexes in IP and whole cell lysates respectively.

The unsuccessful co-IP attempts to verify NS2 interactions with eIF3 θ or eIF4E could be due to numerous factors, including the complexity of conditions required to maintain certain protein complexes in their native interacting conformations. The successful cross-linking and purification of NS2 provides the first steps towards detecting specific NS2 protein interactions in future, that should be possible with further optimisation.

2.4. Discussion

The production of viral replication factories is essential for the efficient replication of many viruses. In addition to housing viral proteins and nucleic acids necessary for the assembly and replication of progeny virions, some replication factories have also been shown to interact with host proteins. Of particular interest to us was the realisation that a broad range of viruses sequester cellular translational machinery within cytoplasmic replication factories to promote viral protein synthesis (Katsafanas and Moss 2007; Sánchez *et al.* 2013; Desmet *et al.* 2014; Schoelz and Leisner 2017). Thus far, the viral inclusion bodies (VIBs) of African horse sickness virus (AHSV) have been described as merely being the sites for core packaging and genome replication (Kar *et al.* 2007). To our knowledge, nothing has yet been reported on the interaction of AHSV VIBs with host proteins. As much is yet to be elucidated about the mechanisms whereby AHSV annexes host protein synthesis machinery to enhance the viral life cycle, investigating the potential involvement of the VIBs in this process was the main focus of this study.

A comprehensive overview of the progression of AHSV VIBs at different stages of the replication cycle was required before exploring potential additional functions of the VIBs. The VIBs were shown to increase in both abundance and size as infection progressed, from 4 h pi onwards. Those sized 1 μm^2 or larger reached a maximum abundance at 24 h pi, after which numbers remained unchanged. This was relatively similar to what was reported for bluetongue virus (BTV) VIBs by Brookes *et al.* (1993), showing the numbers of the VIBs to increase from 6 h pi until approximately 20 h pi, after which numbers stabilised. The sizes of the BTV VIBs between 20 and 28 h pi ranged between 0,7 and 1,1 μm^2 . Our micrographs also revealed substantial levels of cell death from 36 h pi onwards. These results allowed the most suitable time point to be identified at which to conduct downstream analyses, i.e. 24 h pi, where the VIBs are large whilst cells are adequately viable to support a viral infection.

Confocal micrographs showed the VIBs formed by AHSV-4, AHSV-5 and BTV-10 to be morphologically similar, revealing characteristic large, spherical-shaped cytoplasmic foci. In most cases the VIBs, especially those of very large sizes, showed ring-like appearances, with NS2 labelling strongly at the margins and weaker at the centres of the VIBs. This pattern has previously been seen in confocal microscopy images of BTV VIBs, reovirus VFs and rotavirus viroplasms (Kar *et al.* 2007; Touris-Otero *et al.* 2004; Eichwald *et al.* 2018). TEM analyses of AHSV and BTV VIBs have however, revealed a homogenous spread of NS2 throughout the VIB matrix, as is

the case for rotavirus viroplasms and reovirus viral factories (Brookes *et al.* 1993; Uitenweerde *et al.* 1995; Eichwald *et al.* 2012; de Castro *et al.* 2014). Thus, it is likely that the partial labelling of NS2 seen in confocal micrographs is due to insufficient antibody penetration or binding within the dense matrix of the VIBs.

The construction of a pHCMV-NS2-eGFP plasmid expressing fluorescent NS2-eGFP allowed for the detection of NS2 foci in live cells. The specificity of fluorescence to NS2-eGFP foci was confirmed by the detection of NS2 coinciding with eGFP fluorescence in each case. The distribution of eGFP throughout the VIB matrix, compared to NS2 detection restricted to the VIB margins, strongly suggests the latter appearance being a product of insufficient antibody penetration. Live cell analyses provided the first preliminary description of the formation of orbivirus VIBs from precursor NS2 foci. The NS2-eGFP foci were shown to start off as small and dispersedly distributed, and showed rapid, directionless movements. The NS2-eGFP foci in close proximity to one another seemed to eventually coalesce to form larger, more stable aggregates or VIB precursors. These results are similar to the real-time characterisation of rotavirus viroplasms and reovirus VFs (Eichwald *et al.* 2012; Bussiere *et al.* 2017). These results suggest that the movements of NS2 foci towards one another occur in a random fashion and that they merge to progressively form the VIBs. As cells often underwent cell death before the formation of mature VIBs could be documented, our analyses had to be restricted to the early formation of VIB precursors. The sample sizes were however increased by documenting various areas of the cell plate. Continuing the search with more biological replicates in the future should reveal the more advanced stages of VIB formation. Contraction and expansion of the cytoplasm, causing inconstant positions in the z-axes, provided challenges in maintaining focus on the NS2-eGFP foci, however this was mitigated by applying definitive focus to the microscope settings. Combining fast paced Z-stacks with live-cell imaging could provide a possible solution in distinguishing between foci at different focal planes.

The chemical ribopuromycylation assay developed by David *et al.* (2012) allows for the detection of actively translating ribosomes. Conventional CLSM sample preparation (fixation with PFA-PBS followed by Triton-X permeabilisation) resulted in excessive background and below optimal specific signal for PMY. However, concurrent permeabilisation and fixation with buffers that inhibit polysome degradation, as done by Desmet *et al.* (2014), resulted in an optimal signal-to-noise ratio for PMY. The finding that PMY formed large aggregates of bright intensities colocalising with the VIBs of AHSV-4, AHSV-5 and BTV-10 indicated the potential compartmentalisation of protein synthesis within the VIBs. PMY showed a relatively even distribution throughout the VIBs, and plot intensity profiles confirmed the intensities within the VIBs to be higher compared to the diffusely distributed PMY signal throughout the rest of the cytoplasm. These findings were similar to those of Desmet *et al.* (2014), who showed strong colocalisation patterns of PMY with the viral factories of reovirus. We also established that the PMY signal intensities within VIB-like structures, formed by recombinantly expressed NS2, was not elevated compared to the signal in the surrounding cytoplasm. These VIB-like structures are assembled from NS2 in the absence of other viral proteins or nucleic acids, indicating that PMY only labels positively when all of the components essential for the execution of

translation are present. The VIBs contain an abundance of replicatory and transcriptionally active cores, synthesising viral RNAs (Brookes *et al.* 1993; Kar *et al.* 2007). Thus, the elevated levels of PMY within the VIBs suggest that the VIBs also act as hubs for viral translation, hence allowing an efficient mechanism for up-regulating virus protein synthesis. This was a novel finding for any orbivirus.

Understanding how translation is orchestrated in the VIBs required the characterisation of the distribution profiles of different components of the host translation apparatus to the VIBs. The components and sequence of events involved in cap-dependent translation initiation have been reviewed (Shirokikh and Preiss 2018). In brief, eukaryotic translation initiation factor (eIF) 4E binds directly to the 5' cap on mRNAs, and joins with the scaffold protein eIF4G and the RNA helicase protein eIF4A, together forming the eIF4F complex. The small 40S ribosomal subunit is bound to the tRNA^{Met}-eIF2-GTP ternary complex facilitated by eIF1, eIF3 and eIF5, together forming the 43S pre-initiation complex (PIC). eIF3 facilitates the binding of the tRNA^{Met} and the mRNA to the 40S ribosome to form the 43S PIC, and prevents premature joining of the 40S and 60S subunits (Damoc *et al.* 2007). The subunit eIF3θ (also known as eIF3a) forms part of the core complex of eIF3. The 43S PIC scans along the mRNA until the first AUG start codon is reached. The 60S ribosomal subunit ultimately joins, and the eIFs are replaced by elongation factors to complete the synthesis of the polypeptide.

When assessing whether different components of the host translation apparatus were found within the VIBs, it was interesting to observe that the components tested localised to distinct sub-structural domains of the VIBs. Whereas the 60S ribosomal protein L11 and translation initiation factor eIF3θ localised throughout the VIBs, the 40S ribosomal subunit protein S3 and translation initiation factor eIF4E were restricted to the VIB perimeters. As active protein synthesis was shown to occur throughout the VIBs, the localisation of L11 and eIF3θ throughout the VIB was expected. However, for the same reason, the absence of S3 and eIF4E from the central regions of the VIBs was surprising, and the reason for this therefore requires further investigation. VIB-like structures formed by recombinantly expressed NS2 showed a similar distribution pattern for L11, S3, eIF3θ and eIF4E as seen in the context of an AHSV infection. This indicates that the localisation profile of these components to the VIBs is solely mediated by NS2.

One hypothesis to consider is that distinct sub-structural domains of the VIBs relate to specific functions regarding the orchestration of translation. A potential model to describe our results is that cap recognition by the 40S ribosomal subunit (with S3) and eIF4E occurs at the VIB perimeters, and that the components are subsequently trafficked to the centres of the VIBs for translation initiation to occur. This would also explain the presence of eIF3θ and L11 throughout the VIBs, as eIF3θ remains attached to the 43S PIC through the start codon scanning process, and the 60S ribosomal subunit joins at the AUG codon and remains attached for the entire duration of polypeptide synthesis. However, a shortcoming in this model is that it could not explain why S3 is absent from the central regions, as the 40S ribosomal subunit also remains attached to the mRNA until translation is completed (Shirokikh and Preiss 2018). A similar distinctive sub-structural

localisation pattern was reported by Desmet *et al.* (2014) for translation components relative to reovirus VFs. Interestingly, they showed that, despite observing active translation throughout reovirus VFs, L11, S3 and eIF4E colocalised with the inner matrix proteins μ NS of the VFs, whereas eIF3 θ localised to σ NS, which is mostly concentrated at the outer margins of the VFs. Of our results, only L11 thus showed a similar distribution profile to Desmet *et al.* (2014) relative to the VIBs, with the other components showing the opposite distribution profile. Desmet *et al.* (2014) also provided a potential biological model for their observations and proposed differential functions relating to distinct sub-domains of reovirus VFs.

Another possible hypothesis is that S3 and eIF4E are absent from the VIB centres because they might not be required by the virus to execute translation initiation. However, it is assumed that only viruses that do not contain 5' capped RNAs and thus initiate translation at an IRES are capable of initiating translation in the absence of cap-binding initiation factors (Jaafar *et al.* 2019). Orbivirus RNAs are, however capped at the 5' end by the VP4 enzyme contained within the transcriptionally active cores (Ramadevi *et al.* 1998), and thus likely require cap-binding factors like eIF4E to initiate translation. Interestingly however, it has been reported that cap-binding translation initiation factors could down-regulate viral protein synthesis. The eIF4F complex has been shown to up-regulate interferon regulatory factors in the presence of a rotavirus infection, and knock-down of eIF4F components eIF4A, eIF4G and eIF4E was correlated with an increase in rotavirus replication (Chen *et al.* 2019). This suggests the capability of certain viruses with 5' capped RNAs like rotavirus (Imai *et al.* 1983) to initiate translation without requiring the cap-binding translation initiation components. It would be interesting to determine whether the replication cycle of AHSV is affected by the absence of these translation factors. Revealing their non-requirements for AHSV translation could provide another explanation for the absence of eIF4E from the VIB central regions.

Insufficient antibody penetration, or unavailability of their antigen targets, especially in dense matrices like the VIBs, could also explain the appearance of distinct sub-structural localisation patterns of the translation components. TEM images done in the past have shown NS2 distributing homogeneously throughout the VIBs, and previous confocal micrographs have shown partial labelling patterns of NS2, with lower intensities at the centres of the VIBs (Brookes *et al.* 1993; Uitenweerde *et al.* 1995; Kar *et al.* 2007). The possibility for some of the antibodies specific to the translation components tested to have showed partial labelling patterns due to insufficient antibody penetration should thus be considered.

As a start to determine whether there are direct protein-protein interactions between NS2 and the translation factors investigated here, NS2 was cross-linked to maintain interacting proteins within their complex formations. In this study, cross-linking with formaldehyde led to a mobility shift of NS2, as indicated by the higher molecular weight bands on the Western blots for NS2. Following immunoprecipitation, NS2 was maintained in complex formation with other proteins, as similar high molecular weight bands were

detected after pull-down with protein A beads. Desmet *et al.* (2014) used formaldehyde to cross-link σ NS protein interactions with translation factors.

Different cross-linking reagents exist, which each cross-link at distinct distances between interacting partners (Wong and Wongt 1992). The characteristics of formaldehyde as a cross-linker have been reviewed (Srinivasa *et al.* 2015). Formaldehyde is a membrane permeable reagent, and can cross-link proteins in close proximity to one another (2.3 Å). It also has a minimal effect on antigens, making it a suitable cross-linker to use for immunoprecipitation.

Uitenweerde *et al.* (1995) showed higher molecular weight bands for NS2 on Western blots with samples treated in the absence of a reducing agent, which potentially represent NS2-NS2 multimers. Our results showed additional bands to Uitenweerde *et al.* (1995), suggesting that protein interactions other than NS2 with itself were cross-linked. It was aimed to identify whether protein-protein interactions with NS2 mediate the interactions of the VIBs with the host translation apparatus, by immunoblotting for eIF3 θ or eIF4E after cross-linking and immunoprecipitating NS2. However, no bands were detected for these proteins. One possible explanation is that protein interacting domains of eIF3 θ or eIF4E with NS2 is the same epitope regions recognised by their antibodies, resulting in their non-detection due to complex formations with NS2. As protein interactions are highly sensitive to physiological conditions, it is also possible that during any step of sample preparation, unideal physiological conditions lead to the disruption of native protein conformations. As immunoprecipitation was done from whole-cell lysates, the presence of competitor proteins could have diluted the presentation of specific protein interactions of interest. Alternatively, the precipitated protein quantity was below the detection levels of our system. Western blotting could also involve challenges of proteins not being fully transferred to membranes. A potential future experiment which will avoid some of these challenges is the use of an *in vitro* proximity fluorescence assay like Alpha (Amplified Luminescent Proximity Homogeneous Assay) technology. This assay involves incubation of two purified proteins with their specific antibodies, the latter of which are designed to emit fluorescence only when in close proximity to one another. With formaldehyde treatment, protein charges are not affected, and minimal side reactions occur, making it suitable for mass spectrometry (MS) studies (Srinivasa *et al.* 2015). By subjecting NS2 protein complexes maintained by paraformaldehyde to MS, the proteins with which it interacts could be identified.

Numerous other specialised mechanisms exist whereby viruses interact with host translational machinery to their advantage. Swine fever virus non-structural protein NS5A interacts with eIF3 subunit E, thereby sequestering it and up-regulating virus translation (Liu *et al.* 2018). No other orbivirus proteins have been reported to interact with components of the host protein synthesis apparatus, and is thus worthwhile to investigate. A common antiviral stress response is the re-localisation of translation factors and other essential proteins to stress granules (SGs) or P-bodies, where they are kept in an inactive state. Viruses like reovirus

and rotavirus have however been shown to compartmentalise proteins that form SGs and P-bodies to the viroplasms to either disrupt their formation, or to annex components that would have been contained in the SGs to their own use (Carroll *et al.* 2014; Choudhury *et al.* 2017; Dhillon and Rao 2018). It would be interesting to see whether AHSV VIBs interact with components that form SGs or P-bodies.

The results obtained in this study indicated that orbivirus VIBs are involved in more processes than previously anticipated. This is the first evidence that orbivirus VIBs are able to re-localise host proteins, to become hubs of virus protein synthesis. The finding of the ribosomal components and translation initiation factors localising to distinct sub-structural domains of the VIBs is interesting, and the reasons should be further investigated. These results also provided the first preliminary description of the formation of the VIBs from precursor foci. Together, these descriptions of the VIBs provide a better understanding of how AHSV enhances the viral replication cycle.

Future perspectives could include gaining a better understanding of the exact movements and whereabouts of NS2 foci preceding their merging to form the VIBs. This could be done by applying a particle-tracking algorithm to quantify the frequency and directions of their movements before and after coalescence. As a future step following the detection of active protein synthesis in the VIBs, ribosome profiling could be conducted to specifically identify which RNAs are translated in the VIBs. Immunogold labelling TEM analyses could establish whether the localisation profiles observed for the translation factors with confocal microscopy is true, or merely a product of insufficient antibody penetration. It would be worthwhile determining the localisation profiles of additional translation factors, to gain a broader understanding of how translation is orchestrated in the VIBs.

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