

Comparative localisation and trafficking of wild-type and mutant NS3 proteins of African horsesickness virus

by

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DECLARATION

I, Soné Louise Ungerer, hereby declare that the thesis/dissertation that I herewith submit for the degree *Magister Scientiae* to the University of Pretoria contains my own work and has not been previously submitted by me for a degree to this or any other tertiary institution.

Soné Louise Ungerer

Date

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SUMMARY

PROJECT TITLE: Comparative localisation and trafficking of wild-type and mutant NS3 proteins of African horsesickness virus

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African horsesickness (AHS) is a non-contagious, infectious illness of equids, caused by African horsesickness virus (AHSV). The disease causes symptoms ranging from mild fever to severe infection and death in its mammalian host, whilst the insect vector that transmits the virus remains unaffected. The AHSV protein believed to play an important role in cytotoxicity and pathogenesis is the non-structural protein NS3. In Bluetongue virus (BTV) it has been shown that the N- and C-terminal domains of NS3 interacts with VP2 on the outer capsid of newly formed virions, whilst the C-terminal interacts with host cell proteins in order to establish trafficking to the plasma membrane. Once at the cell membrane, virions are released from host cells either through viral budding or by means of cell lysis depending on the host cell type.

Previous studies have identified a number of conserved regions in the NS3 protein. In this study, various mutant AHSV NS3 constructs were compared through membrane association assays and subcellular localisation and trafficking. Some mutants contained substitutions in the conserved regions of the protein to determine the role of these regions in protein trafficking whilst other NS3 constructs contained an eGFP tag on either the N- or C-terminus of the protein to establish the effects of the reporter molecule on localisation and trafficking. All of the mutant proteins showed altered localisation patterns, confirming that these regions play an important functional role in NS3 protein trafficking. Surprisingly, not all the eGFP proteins localised the same as their non-eGFP counterparts suggesting that caution should be taken when studying localisation with reporter molecules.

Colocalisation assays were performed with NS3 and a number of cell organelle markers in order to determine if NS3 uses the cell secretory pathway for trafficking to the plasma membrane. In this pathway, membrane proteins are inserted into the lipid bilayer of the endoplasmic reticulum and

buds from one organelle to the next until the protein reaches its target destination (plasma membrane). It was found that NS3 partially colocalises with the Golgi apparatus which suggest that NS3 traffics through this organelle toward its target destination. By means of pathway inhibition of the ER to Golgi transport, NS3 trafficking to the cell membrane was repressed confirming that NS3 uses the cell secretory pathway. This was found in both insect and mammalian cell lines.

ABBREVIATIONS

°C	degrees Celsius
µl	microlitre
µm	micrometre
Å	angstrom
aa	amino acid
AHS	African horsesickness
AHSV	African horsesickness virus
BHK	baby hamster kidney
bp	base pairs
BTV	Bluetongue virus
CPV	canine parvovirus
Da	Dalton
DAPI	4'6'-diamidino-2-phenylindole
ddH ₂ O	double distilled water
Dept	department
DLP	double layered particle
DNA	deoxyribonucleic acid
ds	double stranded
EDTA	ethylenediaminetetra-acetic acid
EEV	equine encephalosis virus
eGFP	enhanced green fluorescent protein
ER	endoplasmic reticulum
FCS	foetal calf serum
Golgi	Golgi apparatus
HD	hydrophobic domain
HIV	human immunodeficiency virus
hpi	hours post infection
HSV	herpes simplex virus
HygB	Hygromycin B
IP ₃	inositol 1,4,5-triphosphate
IPTG	isopropyl-β-D thiogalactopyranoside
kDa	kilo Dalton
LB	Luria-Bertani medium
M	Molal
mA	milliAmpere
min	minutes
ml	millilitre
mM	millimolal
mm	millimetre
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
MVB	multivesicular bodies
ng	nanograms
nm	nanometer
NS	non-structural
NSP	non-structural protein
NTP	nucleotide triphosphates
OD ₆₀₀	optical density at 600 nanometres
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
Pfu	plaque forming units
PLC	phospholipase C
pmol	picomole

PPI	inorganic pyrophosphate
PSB	protein solvent buffer
RE	restriction enzyme
rER	rough endoplasmic reticulum
RNA	ribonucleic acid
ROI	region of interest
rpm	revolutions per minute
s	seconds
SDS	sodium dodecyl sulphate
Sf9	<i>Spodoptera frugiperda</i> insect cells
SNARE	soluble NSF attachment receptor protein
ss	single stranded
TC	transcription complex
TEMED	N,N,N',N'-tetramethylethylene diamine
TGN	trans-Golgi network
TLP	triple layered particle
TM	transmembrane
U	units
UHQ	ultra high quality distilled water
UV	ultra violet
V	volts
v/v	volume per volume
VIB	viral inclusion bodies
VLP	virus-like particles
VP	virus protein
Vps	vacuolar protein sorting
w/v	weight per volume
WT	wild type
X-Gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

LIST OF BUFFERS

Hypotonic buffer:	10 mM Tris, 0.2 mM MgCl_2 , 1 mg/ml Pefabloc (Roche) 0.7 $\mu\text{g/ml}$ Pepstatin (Roche) [pH 7.4]
NTE:	100 mM NaCl, 10 mM Tris, 1 mM EDTA [pH 7.4]
PBS:	137 mM NaCl, 2.7 mM KCl, 4.3 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 1.4 mM KH_2PO_4 [pH 7.3]
PSB (2x):	125 mM Tris-HCl 4% SDS, 20% glycerol, 10% 2-mercaptoethanol [pH 6.8]
TAE buffer:	40 mM Tris-HCl, 20 mM Na-Acetate, 1 mM EDTA
TGS buffer:	25 mM Tris-HCl, 192 mM glycine, 0.1% SDS [pH 8.3]
Transfer buffer:	0.025 M Tris, 0.15 M glycine, 20% methanol [pH 8.3]

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Chapter 1:

Literature Review

1.1 Introduction

African horsesickness virus (AHSV) is the causative agent of a disease with the same name, African horsesickness (AHS), a non-contagious, infectious disease of mainly equids (Mellor, Hamblin, 2004). Although AHSV infects mainly equids, AHS has occasionally been described in elephants and camels as well as in African carnivores (Alexander *et al.*, 1995) and it has been shown to be fatal in dogs that have ingested infected horsemeat (Bevan, 1911). This arthropod-borne disease is transmitted to its mammalian host through *Culicoides* midges (Diptera: Ceratopogonidae). The midges obtain the virus after a blood meal on an infected horse and become infective after approximately twelve days. The virus can then again be transmitted to susceptible horses with another 12 days incubation time in the new host prior to the onset of viraemia (Mellor, Hamblin, 2004).

Most experimental research on transmission of AHSV has been performed using *Culicoides variipennis* as a model (Boorman *et al.*, 1975), however other species have been shown to be susceptible to specific serotypes of AHSV. These include *C. engubandei*, *C. magnus*, *C. zuluensis*, *C. pycnostictus*, *C. bedfordi* and *C. dutoiti* from the Old World species (Paweska *et al.*, 2003). Even though all the above-mentioned species can act as carriers of AHSV, *C. imicola* (Venter *et al.*, 2005) and the more recently identified *C. bolitinos* are the major vectors in field transmission (Meiswinkel, Paweska, 2003).

Upon infection of the mammalian host, the virus multiplies in the lymph nodes after which it migrates to the target organs (heart, lungs and spleen) (Clift, Penrith, 2010; Erasmus, 1973), therefore AHS affects the circulatory and respiratory systems (Mellor, Hamblin, 2004). AHS has been classified into four clinical forms dependant on the duration and severity of infection (Theiler, 1921). In ascending order of severity they are: (1) AHS fever; (2) the acute or mixed form; (3) subacute or oedematous form also described as the “dikkop” form; and (4) the peracute/pulmonary or “dunkop” state. The latter form of the disease is responsible for most deaths in infected animals with a mortality rate of up to 95% (Mellor, Hamblin, 2004). Because of the high mortality rate, AHS has been assigned OIE (Office Internationale des Epizooties) former List A Status (Mellor, Hamblin, 2004).

The first reported outbreak of AHS occurred in Yemen in 1327 (Bourdin, 1973), however it is believed that the virus originated in Africa (Swanepoel *et al.*, 1992). Since the recognition of the disease in 1719 (Erasmus, 1964), there have been a number of outbreaks in southern Africa of which the most devastating occurred in 1854/1855. During this outbreak, 70 000 horses died as a result of AHS (Barnard, 1998; Coetzer, Erasmus, 1994). An outbreak in 2004 in the Western Cape surveillance area, a previously AHS-free area, led to a two year deferment on the export of horses

(Venter *et al.*, 2006). These strict quarantine regulations can have a great economic impact on affected countries regarding the racing and show jumping industries. Since 1998, 15 northern European countries have experienced bluetongue disease (caused by bluetongue virus, a sister virus to AHSV) outbreaks for the first time (Purse *et al.*, 2005) with the most recent initial outbreaks in four central European countries in 2006. It is believed that these outbreaks are linked to the overall rise in temperature in Europe. The main identified vectors of bluetongue virus (BTV) include a number of *Culicoides* species, which increases the fear for other viral outbreaks like AHS which is transmitted by the same vector (Gale *et al.*, 2010; MacLachlan, Guthrie, 2010; Mehlhorn *et al.*, 2007).

To date, nine different AHSV serotypes have been identified, all belonging to the *Orbivirus* genus, a member of the family *Reoviridae* (Mellor, Hamblin, 2004). The family *Reoviridae* is one of six dsRNA virus families. It currently contains the largest number of genera, which infect the most diverse host range (including vertebrates, mammals, insects and plants) of all the families. Classification of viruses into the *Reoviridae* family rests upon three features. The genetic material must consist of double stranded RNA (dsRNA); the genome is usually segmented into 10, 11 or 12 segments each of which codes for 1-3 proteins on only one of the complementary strands; the virus is non-enveloped thus it does not contain a lipid bilayer (Knipe, Howley, 2004). The virion sizes range between 60-85 nm and the capsid proteins are arranged to reflect an icosahedral symmetry.

The focus of this study is on the AHSV non-structural protein NS3. NS3 has been shown to be responsible for the cytotoxic effects in infected host cells (Stoltz *et al.*, 1996; Van Staden *et al.*, 1995) and plays an important role in pathogenicity of AHS. To date very little is known about the trafficking and localisation of this protein in neither insect nor mammalian cells. This literature review will give an overview of the virus structure and genome organization, the role of BTV NS3 (the prototype orbivirus), the rotavirus NSP4 (proposed analogue of NS3), intracellular trafficking and the development of the confocal microscope and its application to studying intracellular trafficking and localisation.

1.2 Virus structure and genome organization

Of the nine genera in the *Reoviridae* family, the *Orbivirus* genus is the most important in animal diseases. There are 19 identified serogroups of orbiviruses. Both bluetongue virus (BTV) and African horsesickness virus (AHSV) are members of the *Orbivirus* genus within the *Reoviridae* family (Bremer, 1976, Oellermann *et al.*, 1970). Bluetongue virus (BTV) is the prototype member of the orbiviruses within the *Reoviridae* family and therefore the virion structure will be described in terms of BTV.

Similar to all *Reoviridae* members, BTV is a non-enveloped virus with two protein shells consisting of seven structural proteins (VP1-VP7). The outer protein shell is referred to as the capsid and the inner protein shell as the core. The capsid contains two major proteins VP2 and VP5 (Verwoerd *et al.*, 1972; Verwoerd *et al.*, 1970). The VP2 proteins have a triskelion shape and VP5 a globular shape and the organization of these proteins in the capsid gives it an icosahedral configuration. The VP2 protein has receptor-binding properties for cell entry upon infection and has also been described as a viral haemagglutinin (Cowley, Gorman, 1987; Hassan, Roy, 1999) whilst the VP5 protein plays a role in cell penetration (Hassan *et al.*, 2001). Both the VP2 and VP5 capsid proteins are in contact with the VP7 protein trimers of the core (Figure 1).

The capsid can be easily removed from the virion (Huisman *et al.*, 1987b) to reveal the core structure of approximately 69 nanometres (nm) in diameter. The core also has icosahedral symmetry with a triangulation number of 13 ($T=13$) in a left-handed configuration. The core has two protein layers that consist of VP7 and VP3 and encapsidates the BTV genome. The outer layer of the core consists of VP7 trimers arranged as six-member and five-member rings. There are 260 VP7 trimers per core particle (Prasad *et al.*, 1992), which give the core a ring-like structure (*orbis* = ring) from which the genus name was derived.

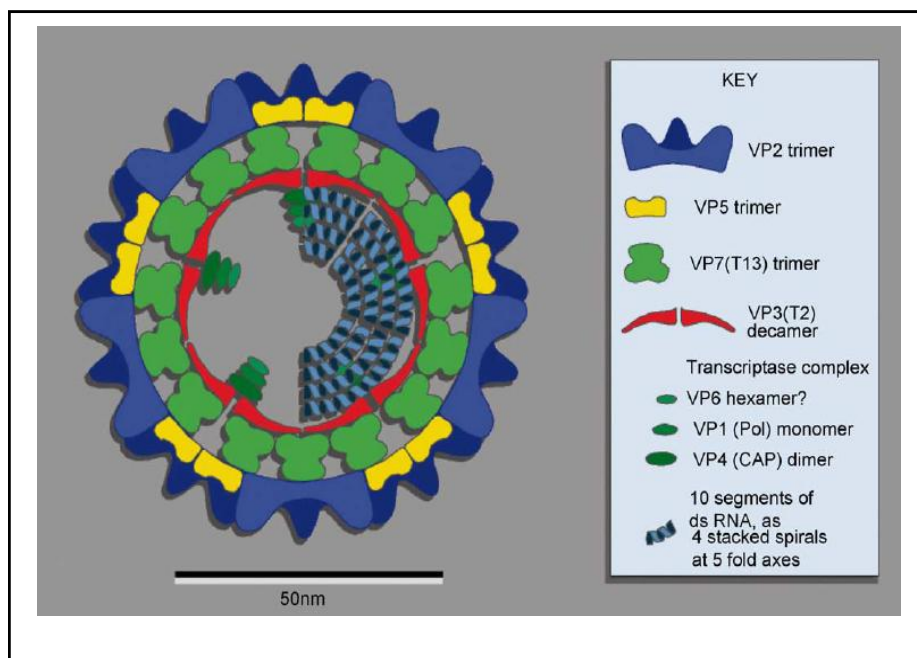


Figure 1. Schematic diagram of the structural proteins in a virus particle (Mertens *et al.*, 2000).

VP7 trimers have a top and bottom domain linked by very weak hydrophobic and specific hydrogen bonds (Basak *et al.*, 1996). The top domain interacts with VP5 and the bottom domain interacts very closely with VP3 on the inner layer of the core (Basak *et al.*, 1996). There are 120 molecules of VP3 protein in the core that are arranged as two sets (A and B) of dimers to provide the scaffold

for the VP7 layer (Grimes *et al.*, 1997). The configuration of the A and B molecules gives the VP3 protein a disk-shaped appearance. Twelve VP3 decamers are arranged from end to end to make up the inner layer of the core. VP3 interacts with the minor proteins in the central density of the virion (Tanaka, Roy, 1994). The central portion of the virion contains three minor proteins, VP1, VP4 and VP6 as well as genomic RNA (Figure 1).

One of the characteristics of BTV that led to its classification as a member of the *Reoviridae* family is its segmented dsRNA genome (Verwoerd *et al.*, 1970). The genome consists of 19 219 base pairs (bp) of dsRNA which is divided into 10 segments. The sizes of the each of the 10 segments range between 822 - 3944 bp (Maan *et al.*, 2008) with the genome having a total molecular weight of approximately 1.5×10^7 Da (Verwoerd *et al.*, 1970). Whole genome sequence comparisons between AHSV, BTV and equine encephalosis virus (EEV) revealed that the genes of all these viruses have the same conserved sequences at their 5' (GUU) and 3' (UAC) termini (Potgieter *et al.*, 2002).

Within the virion approximately 80% of the genome is arranged as four concentric layers with a 26-30 Å centre-to-centre spacing between the RNA strands (Roy, 2005). A model for the genome organization has been derived from X-ray crystallography studies, which suggest that the A and B molecules of VP3 form grooves with which the outer ring of the dsRNA interacts (Mertens, Diprose, 2004). The VP1, VP4 and VP6 structural proteins also interact with the genomic dsRNA rings. As the RNA molecules pass inward, the density and symmetry decrease, however the spiral organization of the genome is maintained (Gouet *et al.*, 1999).

The 10 dsRNA segments (numbered from segment 1 to segment 10 (Seg-1 to Seg-10) arranged in order of decreasing size according to their migration profile on an agarose gel (Bremer *et al.*, 1990; Mellor *et al.*, 2009) each codes for a single specific viral protein except for segment 10 that encodes two related proteins (Figure 3). As a result, seven structural proteins VP1-VP7 and four non-structural proteins, NS1, NS2, NS3 and NS3A are produced in infected cells (French *et al.*, 1989; Mertens *et al.*, 1984). This information is summarised in Table 1.

Table 1: A summary of the 10 BTV genes, the proteins they encode and the protein functions.

Genome segment	Protein	Gene length (bp)	Number of amino acids	Molecular weight (kDa)	Function of proteins
Seg-1	VP1	3954	1302	149	RNA dependent RNA polymerase
Seg-2	VP2	2926	956	111	Cell attachment protein
Seg-3	VP3	2772	901	103	Organization of capsid proteins
Seg-4	VP4	2011	644	76	Capping enzyme
Seg-5	NS1	1770	526	59	Forms tubules during replication
Seg-6	VP5	1639	522	64	Cell entry
Seg-7	VP7	1156	349	38	Cell entry
Seg-8	NS2	1123	357	41	Forms Viral Inclusion Bodies
Seg-9	VP6	2046	329	35	Helicase
Seg-10	NS3	822	229	25	Glycoproteins believed to play a role in cell exit and determine virulence
Seg-10	NS3A	822	216	24	

(Compiled from Mertens *et al*, 1984; French *et al*, 1989; Mellor *et al*, 2009)

1.3 Virus Replication

In order to enter the host cell, the virus depends on mechanisms that involve the recognition and binding of viral receptors (VP2) to generalised proteins such as heparin sulphate which then bind to the host cell receptor, normally a glycoprotein (Forzan *et al.*, 2004; Hassan, Roy, 1999). In a recent study it was found that the virus can enter mammalian host cells through clathrin and cholesterol independent mechanisms that resemble macropinocytosis (Gold *et al.*, 2010). Up until then it was suggested that BTV enters mammalian host cells mainly by clathrin-mediated endocytosis (Forzan *et al.*, 2007). Inside the endosome, pH changes result in the degradation of VP2 on the outer capsid to expose the VP5 proteins. The VP5 protein then fuses with the endosomal membrane (Forzan *et al.*, 2004) and induces permeabilisation (Hassan *et al.*, 2001) to free the transcriptionally active core particle into the host cell cytoplasm (Diprose *et al.*, 2001). Once inside the Mg^{2+} -rich cytoplasm, the core expands around the 5-fold axes (Gouet *et al.*, 1999). This allows the core particle to be more permeable to nucleotide substrates (NTPs necessary for transcription) and ions, which enter the core, and for the release of mRNAs and by-products into the cytoplasm (Diprose *et al.*, 2001) In *Reoviridae*, all the genome segments are transcribed simultaneously (Huisman, Verwoerd, 1973).

When a mammalian cell encounters dsRNA in its cytoplasm, an immune response is evoked. These defence responses involve the modification of the host cell translation control mechanisms to induce apoptosis in order to prevent spread of the foreign material. To promote viral replication and avoid host cell shutdown, the virus replication takes place within the core particle so that

dsRNA never comes into contact with the host cell (Eaton *et al.*, 1990). It has also been shown that the BTV core particle can bind dsRNA from damaged viruses in the cell to prevent it from inducing host cell defence mechanisms (Diprose *et al.*, 2002).

Three minor structural proteins act together to form the viral transcription complex (TC). VP1 is the viral polymerase (Urakawa *et al.*, 1989), VP4 acts as a capping enzyme (Ramadevi *et al.*, 1998) and VP6 exerts helicase activity (Kar, Roy, 2003; Stauber *et al.*, 1997). VP6 unwinds dsRNA and the Mg²⁺ dependent polymerase (VP1) transcribes the negative sense RNA strand to produce a positive sense mRNA daughter strand and inorganic pyrophosphate (PPi) as a by-product (Diprose *et al.*, 2001). The newly formed daughter strand is then capped and methylated by VP4 (Ramadevi *et al.*, 1998) and ready to exit the core particle. After RNA synthesis has taken place, the parent strands re-anneal and return to their original packaged state (Banerjee, Shatkin, 1970).

The daughter strands exit through the pore in the VP3 and through the pentameric rings of VP7 (P-trimers). These rings are blocked in the intact particle by VP7 T-trimers (Diprose *et al.*, 2001). Inside the cytoplasm, the new mRNAs can be translated to produce new structural and non-structural proteins. Structural proteins can be used to produce new virus particles or together with non-structural proteins, the mRNA's serve as templates for negative strand synthesis to produce dsRNA – the genome of the new particles (Diprose *et al.*, 2001).

As is the case in most organisms, some proteins are produced in abundance whilst others occur in only trace amounts, depending on the function of the synthesized proteins. One protein that is produced in large amounts from BTV transcription and translation is the non-structural protein NS2. The NS2 protein is the only BTV protein that undergoes phosphorylation (Huisman *et al.*, 1987a). Phosphorylated NS2 forms viral inclusion bodies (VIBs) that serve as sites for viral assembly (Modrof *et al.*, 2005). Since the NS2 amino terminus has a high affinity for ssRNA it is believed to recruit new mRNAs to the VIB (Roy, 2005) and evidence suggests that NS2 might also function in RNA packaging (Lymeropoulos *et al.*, 2003; Taraporewala *et al.*, 2001). The VIBs have a perinuclear location inside the cytoplasm (Brookes *et al.*, 1993). VIBs are rich in mRNAs and phosphorylated NS2 (Thomas *et al.*, 1990) and structural proteins VP3 and VP7 that form the cores are also present in VIBs (Kar *et al.*, 2005). The two proteins that compose the outer capsid, VP2 and VP5, are present at the periphery of the VIBs (Hyatt, Eaton, 1988).

Inside the VIB VP1, VP4 and VP6 associate with VP3 decamers. This association starts the assembly of VP3 and initiates core formation. VP7 assembles around VP3 establishing hydrophobic interactions between the VP7 trimers and the VP3 scaffold. Once the core is fully assembled, it is encapsidated by VP2 and VP5 both of which attach to VP7. Once the capsid has assembled, the core is no longer transcriptionally active and the newly formed virus can now be released from the infected cell (Roy, 2005).

1.4 Viral release

Bhattacharya *et al.* (2007) showed that outer capsid protein VP2 interacts with vimentin intermediated filaments. The authors suggest that this interaction plays an important role in viral egress since a disruption in the vimentin filament network reduces the number of viruses that are released from infected cells (Bhattacharya *et al.*, 2007). In both mammalian and insect cells the non-structural proteins NS3 and NS3A are believed to facilitate viral egress from cells. Current investigations are also trying to resolve the role of non-structural protein NS1 in the final stages of the BTV life cycle.

1.4.1 NS1

Expression of the Seg-5 BTV gene in mammalian cells leads to the synthesis of the 64kDa non-structural NS1 protein (Huismans, Els, 1979; Urakawa, Roy, 1988). NS1 is the most abundantly expressed BTV protein (*in vivo*) and is responsible for tubule formation in BTV and AHSV infected cells (Huismans, Els, 1979). These tubules are cytoskeleton associated and have been found to localise around the periphery of VIBs during viral replication (Eaton *et al.*, 1988). Electron microscopy revealed that these BTV NS1 tubular structures are approximately 52 nm in diameter with lengths that can reach up to 1000 nm. The AHSV NS1 proteins are highly conserved (Maree, Huismans, 1997) and produce tubules with a 23 nm diameter with lengths of up to 4 μ m (Van Staden *et al.*, 1998). Owens *et al.* (2004) used a protein knockout approach to study the role of NS1 during the viral replication process. By interfering with NS1 protein function and/or tubule formation four distinct alterations in the viral life cycle could be observed: a reduction in virus-induced cytopathic effects, a 10-fold increase in the number of viruses that was released into the culture medium, a shift from lytic release to budding from the plasma membrane, and fourthly no tubules formed. This led to the conclusion that NS1 might be one of the major determinants of pathogenicity and morphogenesis in vertebrate hosts where high NS1 levels ultimately lead to cell lysis and virion release. In insect cells however, NS1 tubules are also found in abundance but the newly formed virions are still released through viral budding (Owens *et al.*, 2004). Owens *et al.* (2004) proposed that the NS1/NS3 ratio determines the mechanism through which viral progeny is released from infected cells. In mammalian cells where the NS1 levels are considerably higher than the NS3 levels, virus particles accumulate in the cell cytoplasm, which results in cell lysis. In insect cells where the NS3 protein is overexpressed (Guirakhoo *et al.*, 1995) and occur in levels higher than those of NS1, viruses are released through NS3-induced budding. This hypothesis is currently under further investigation (Owens *et al.*, 2004).

1.4.2 NS3

The focus of this study is on the localisation and trafficking of AHSV NS3. The NS3 protein has been extensively studied in BTV in terms of structure, functional domains, cytotoxicity and its

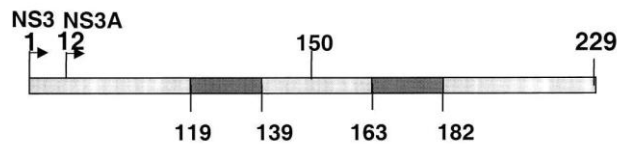
possible role in viral trafficking. The BTV NS3 will therefore be discussed in more detail in close comparison to the research that has been done to date on AHSV NS3.

Unlike all the other BTV dsRNA segments that each code for a single protein, the 822 bp Seg-10 gene codes for two related non-structural proteins, NS3 and NS3A (French *et al.*, 1989; Lee, Roy, 1986; Van Dijk, Huismans, 1988). The alternative NS3A protein is the result of a second downstream initiation codon within the same open reading frame (Gould *et al.*, 1988; Hwang *et al.*, 1992; Lee, Roy, 1986; Wade-Evans, 1990). As a result the 229 aa NS3 protein (25kDa) and a truncated form, the 216 aa NS3A (24kDa) which lacks the first 13 aa are produced (Huismans, van Dijk, 1988; Wu, 1992).

The AHSV S10 nucleotide sequences of all serotypes have also been determined (De Sa *et al.*, 1994; Sailleau *et al.*, 1997; Van Staden, Huismans, 1991). AHSV NS3 is the second most variable protein (VP2 is the most variable) with up to 36.3% variation across serotypes and 27.6% variation within serotypes (Van Niekerk *et al.*, 2001b). The high variability of the S10 gene makes it a useful tool in characterization of different variants in the case of an AHS outbreak (Quan *et al.*, 2008). Sequence analysis of AHSV NS3 showed clustering into three distinct groups (Martin, 1998). BTV NS3 on the other hand is highly conserved, with a maximum of 7% variation (Pierce *et al.*, 1998).

Comparative sequence analysis of six BTV Seg-10 genes revealed a number of conserved sequences that include (a) a single conserved tryptophan at residue 159, (b) two conserved cysteines at residues 137 and 181 and two potential N-linked glycosylation sites at residues 63-65 and 150-152, (c) a cluster of six prolines within a 15 aa region near the N-terminus and (d) the longest 3' non-coding sequence (approximately 113 bases) of all the BTV genes (Hwang *et al.*, 1992). Comparative NS3 sequence analysis of 5 AHSV isolates with other orbiviruses also revealed a number of conserved domains despite the high variability. These include two putative transmembrane hydrophobic domains (TM1 and TM2 - aa residues 116-137 and 154-176 respectively), a cluster of conserved proline residues (aa 22-34) and a highly conserved amino acid region at NS3 amino terminus (Van Staden *et al.*, 1998). Similar to AHSV, BTV NS3 proteins also have two conserved hydrophobic domains (aa 116-137 and 154-176), which are believed to span the plasma membrane (Hyatt *et al.*, 1991; Wu *et al.*, 1992). These hydrophobic domains are arranged in the membrane so that both the N- and C-terminal domains of NS3 project into the cytosol (Bansal *et al.*, 1998). The glycosylation site at residue 150 of BTV NS3 is located between the two hydrophobic domains (Figure 2). Even though all NS3/A proteins have this site, glycosylation does not seem to play a role in protein function since both BTV NS3 and NS3A proteins exist in glycosylated and unglycosylated forms when they are expressed in mammalian cells (Wu *et al.*, 1992). The AHSV NS3/A proteins lack these glycosylation sites which suggest that glycosylation of BTV NS3 might prevent degradation of these proteins without affecting protein function (Bansal *et al.*, 1998; Van Staden, Huismans, 1991).

A



B

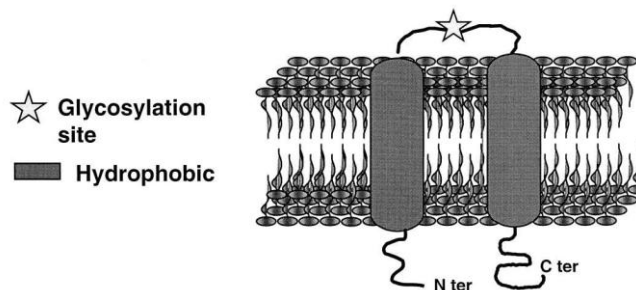


Figure 2. (A) A diagrammatic representation of the BTV NS3 protein with the two initiation codons. The shaded regions indicate the two hydrophobic domains (between aa 119-139 and aa 163-182 respectively) and residue 150 indicates the glycosylation site. **(B)** A diagram which demonstrates the association of NS3 with the membrane (Fields Virology, 2004).

Expression of AHSV in mammalian cells has shown that NS3 and NS3A are synthesized in equimolar amounts (Van Dijk, Huismans, 1988; Van Staden *et al.*, 1995). Similar results were obtained when NS3 was translated in vitro (Van Staden, Huismans, 1991). Analysis of BTV NS3/NS3A levels in infected cells showed that NS3 is the primary product whilst NS3A is only present in trace amounts (French *et al.*, 1989). As previously mentioned, elevated NS3/A levels in invertebrate cells correlates with non-lytic viral release (budding) from cells (Guirakhoo *et al.*, 1995) whilst the lower levels of NS3 in mammalian cells correspond with lysis and cell death (Beaton *et al.*, 2002).

NS3 proteins have been associated with cytotoxicity in BTV (French *et al.*, 1989) and AHSV after expression in mammalian and invertebrate cells (Van Niekerk *et al.*, 2001a; Van Staden *et al.*, 1995). The cytotoxic effect of NS3 depends on the association and topography of the hydrophobic membrane spanning domains in the plasma membrane (Van Niekerk *et al.*, 2001a). It has been shown that NS3 exerts viroporin-like activity in BTV infected cells (Han, Harty, 2004). Viroporins are small hydrophobic transmembrane proteins that can form hydrophilic pores through lipid bilayers. NS3 is a transmembrane protein with two hydrophobic domains associated with the cell membrane. Through the expression of NS3, mammalian cells became more permeable to Hygromycin B (HygB), whilst wild-type cells remained impermeable to HygB. These results indicate that BTV NS3 can play a role in viral release through pore formation that causes destabilisation of the plasma membrane. The first transmembrane (TM1) domain (aa 118-148) was specifically responsible for the viroporin-like activity in BTV (Han, Harty, 2004). Mutation in either of the TM

domains of AHSV prevented the NS3 protein from anchoring in the membrane and abolished the cytotoxicity in infected cells (Van Niekerk *et al.*, 2001a).

A study by Hyatt *et al* (1993) showed that BTV NS3 aids in the release of virus-like particles (VLPs) from infected cells. After yeast two-hybrid studies, Beaton *et al.* (2002) determined that the first 13 amino acids of the NS3 N-terminal folds into an amphipathic helix that interacts with the calpactin (a cellular protein) light chain (p11). The p11 normally binds to the annexin II heavy chain (p36) to form a heterotetramer. The annexin II complex has been shown to play a role in the cellular endocytic and secretory pathways that involves vesicular trafficking (Creutz, 1992; Raynal, Pollard, 1994). As the C-terminal of the NS3 protein binds to the VP2 protein on the outer capsid of the newly assembled virion, NS3 mimics the binding of p36 to p11 and the NS3-p11 complex facilitates exit of the virion across the plasma membrane (Figure 3). NS3 therefore serves as a bridge between the virion and a membrane trafficking protein, to facilitate viral exit (Beaton *et al.*, 2002).

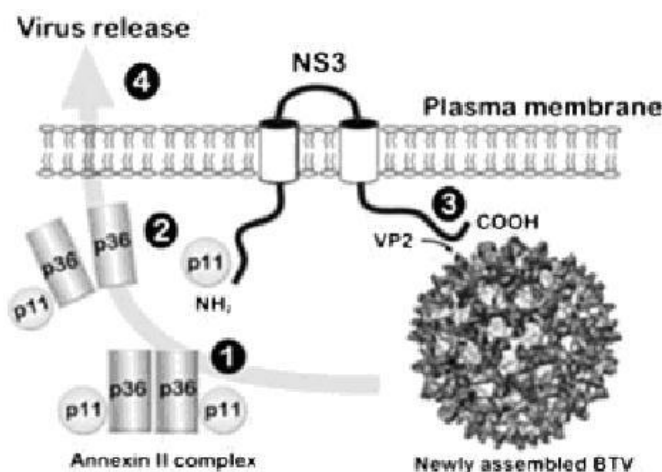


Figure 3. The N-terminal of NS3 binds to the calpactin light chain (p11) and the C-terminal associates with the VP2 protein on virion to facilitate viral extrusion (Beaton *et al*, 2002).

Apart from cell lysis, viruses also use budding as a mode of viral exit from infected cells. Viral budding is the process where an enveloped virus acquires an external layer usually from the host cell membrane that encloses the virion. This newly formed vesicle around the virion is then used to transport the virion out of the cell. Many viruses make use of host-assisted budding in which the virus encodes late domain motifs that enable the virus to exploit the host proteins involved in the vacuolar proteins sorting (Vps) pathway to give rise to multivesicular bodies to aid in viral budding. However, the Vps pathway is not sufficient for all viruses and in the case of Human Immunodeficiency virus (HIV) the virus encodes alternative proteins (Env and Gag) to facilitate budding. A number of enveloped viruses have the ability to exit host cell by means of budding

without actively making use of the Vps pathway (Chen, Lamb, 2007). Three conserved late domain motifs (L-domains - PTAP/PSAP, YPXL/LXXLF and PPXY) have been identified in viral proteins that are associated with budding. The PTAP/PSAP motif interacts with the tumor susceptibility gene (Tsg101) that is a component of the ESCRT-1 complex (Garrus *et al.*, 2001). The ESCRT-1 complex normally plays a role in the formation of multivesicular bodies (MVBs) in the vesicular protein sorting (Vps) pathway associated with viral budding. Recent evidence suggests that BTV might use similar mechanisms to establish viral budding. The PTAP of BTV NS3 recruits the Tsg101 (ESCRT-1) and the YPXL/LXXLF (NS3) motif binds to the AIF-1/Alix (ESCRT-3) to form a bridge between the ESCRT-1 and ESCRT3 complexes (Wirblich *et al.*, 2006). The PPXY motif also recruits host Ubiquitin ligases and even though it is known that these ligases are involved in budding, their exact role in the budding process is not clear (Blot *et al.*, 2004). Since orbiviruses are released from insect cells without distinctive cytopathic effects it is believed that NS3 could exploit the Vps pathway by binding to Tsg101 and recruiting MVB forming machinery for cellular extrusion from insect hosts (Figure 4) (Wirblich *et al.*, 2006).

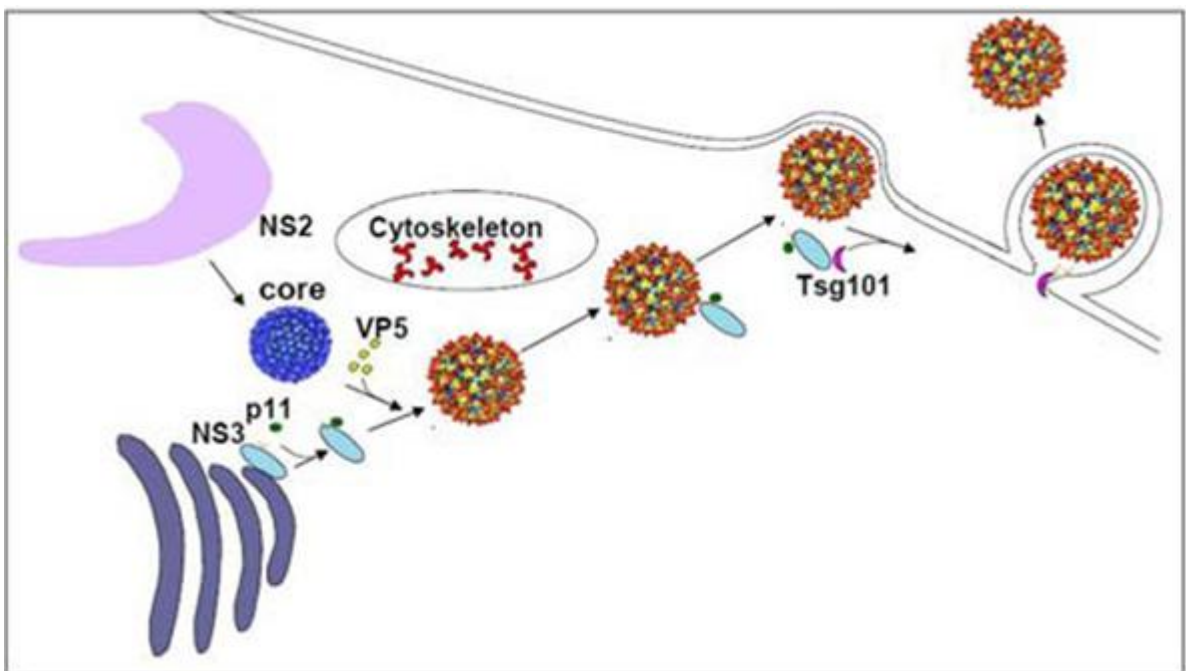


Figure 4. A schematic diagram illustrating the interaction of the NS3 protein with host cell trafficking proteins (p11 and Tsg101) to facilitate viral trafficking and release by means of budding. (Roy *et al.* 2008)

Localisation studies on BTV NS3 and NS3A provided results that both proteins locate to the intracellular smooth-surfaced vesicles such as the endoplasmic reticulum (ER), the Golgi apparatus and the plasma membrane (Hyatt *et al.*, 1991). This localisation suggests that these proteins can be transported between these cellular compartments (Wu *et al.*, 1992) and that the NS3/NS3A proteins are processed in the ER and Golgi apparatus (Bansal *et al.*, 1998). Confocal images of the NS3 in transfected HeLa cells confirmed co-localisation of NS3 with Tsg101 specifically in intracellular compartments. NS3 expression localised to the plasma membrane as

well (Wirblich *et al.*, 2006). These findings indicate that there is a correlation between the associations of viruses with plasma membrane and the localisation of NS3. In both BTV and AHSV the association and localisation of NS3 to the plasma membrane does not require the presence of viral particles (Hyatt *et al.*, 1991; Stoltz *et al.*, 1996).

The molecular structure of NS3 appears to be analogous to that of rotavirus NSP4, a non-structural glycoprotein found in the rough endoplasmic reticulum (rER) membrane which has been shown to function as a viral receptor that binds rotavirus particles and facilitates budding through the rER (Au *et al.*, 1989; Ericson *et al.*, 1983; Kabcenell, Atkinson, 1985; Meyer *et al.*, 1989). Even though localisation of NS3 involves the ER, there is no evidence that NS3 buds through the ER membrane. However NS3 might exert an analogous function to NSP4 in that it facilitates viral trafficking to the plasma membrane. The function of rotavirus NSP4 will therefore be discussed in more detail.

1.5 Rotavirus NSP4 protein

Rotavirus NSP4 protein has been extensively studied and the function of this protein has been very well described. Because NSP4 has been shown to be involved in the cytotoxicity of rotavirus, and because it has been shown to play a role in the trafficking of viral particles to the host cell membrane via a variety of mechanisms, NSP4 is considered to have an analogous function to BTV/AHSV NS3/A. Therefore the role that NSP4 plays in viral release of particles can be considered as a model for the basis of our study.

The *Rotavirus* genus is also included in the *Reoviridae* family (Matthews, 1979). Like orbiviruses, rotaviruses are non-enveloped viruses with a segmented dsRNA genome (Kalica *et al.*, 1978). The 11 segments code for 6 structural and 6 non-structural proteins. Lecce *et al.* (1978) were the first to associate rotavirus infection with fatal diarrhoea in neonatal piglets and soon afterwards the same virus was found to be responsible for infection causing diarrhoea in other animals and humans (Lecce *et al.*, 1978; Saif *et al.*, 1980). Today rotavirus accounts for most cases of gastrointestinal disease in children younger than 5 years of age. Approximately 111 million incidents of gastroenteritis are recorded annually.

The non-enveloped rotavirus particle has a capsid which consists of three concentric protein layers also referred to as triple-layered particles (TLPs). The outermost layer is made up of two structural proteins VP4 and VP7. Upon cell entry, the Ca^{2+} -dependent VP7 layer is degraded due to the low concentration of Ca^{2+} in the cytoplasm resulting in double-layered particles (DLPs). These DLPs consist of two protein layers, a VP2/VP6 layer which encapsidates the VP3/VP1 layer which is in direct contact with the rotavirus genome. DLPs are believed to assemble into viroplasms – electron

dense viral inclusion bodies in which replication takes place inside the cell cytoplasm (Altenburg *et al.*, 1980). These viroplasms are located adjacent to the ER (Petrie *et al.*, 1984). The final step of viral morphogenesis involves the budding of the DLPs through the ER membrane. During the budding event, the outer capsid proteins VP4 and VP7 as well as a temporary ER membrane is acquired.

The rotavirus non-structural glycoprotein, NSP4 (encoded by segment 10) has been described as a multifunctional protein which can act as a viral receptor (Au *et al.*, 1989; Lopez *et al.*, 2005) during viral replication (Berkova *et al.*, 2006; Silvestri *et al.*, 2005). The NSP4 protein was the first described viral enterotoxin (Ball *et al.*, 2005; Parr *et al.*, 2006; Zhang *et al.*, 1998) and is also the protein that facilitates trafficking of particles during viral release (Cuadras *et al.*, 2006; Mir *et al.*, 2007; Storey *et al.*, 2007). NSP4 is a transmembrane protein localised to the rough endoplasmic reticulum (rER) with a 10kDa fraction of the NSP4 carboxy terminus projecting into the cell cytoplasm (Bergmann *et al.*, 1989; Kabcenell, Atkinson, 1985), whilst the N-terminal projects into the ER. The carboxy terminus has been shown to act as a receptor which binds to VP6 proteins situated on the outside of DLPs, and facilitates virus budding across the rER membrane (Au *et al.*, 1989) whilst the N-terminal has a retention signal for the VP7 protein (Maas, Atkinson, 1994). It has been shown that NSP4 has ER membrane destabilisation activity which suggests that this protein might play an important role in the removal of the transient membrane acquired by the DLP during budding (Tian *et al.*, 1996). The removal of the transient membrane is the final step of viral morphogenesis which leads to the production of mature triple-layered particles and it has recently been shown that VP7 is also required for this process (Lopez *et al.*, 2005).

Recently a study by Hyser *et al.* (2010) identified a membrane insertion domain (aa 47 - 90) in the NSP4 protein which allows the protein to facilitate membrane trafficking by acting as a viroporin (Hyser *et al.*, 2010). NSP4 possesses a microtubule-binding domain and functions as a microtubule-associated protein (MAP) to prevent ER-to-Golgi trafficking (Xu *et al.*, 1997). It has been suggested that by blocking the ER to Golgi trafficking NSP4 influences the brush-border associated digestive enzymes and promotes the onset of diarrhoea (Xu *et al.*, 2000). NSP4 interacts with the molecular chaperone calnexin (Mirazimi *et al.*, 1998). Calnexin is an ER-associated chaperone which is believed to play a role in folding and assembly of glycoproteins that pass through the cellular secretory pathway. Other studies have also shown that NSP4 and some of its truncated proteins are released from cells via a Golgi-dependent secretory pathway (Bugarcic, Taylor, 2006; Mirazimi *et al.*, 2003; Zhang *et al.*, 2000). However recent studies found that the NSP4 might use a vesicular pathway which involves travelling via the cytoplasm to vacuole/lysosome to cell membrane pathway which bypasses the Golgi apparatus (Mir *et al.*, 2007; Storey *et al.*, 2007). NSP4 directly associates with the membrane trafficking protein caveolin-1 which facilitates the intracellular trafficking of NSP4 from the ER to the cell surface (Parr *et al.*,

2006; Storey *et al.*, 2007). It has been proposed that even though mature virions do not pass through the Golgi, they do transit through an intermediate compartment between the ER and the Golgi complex (Cuadras *et al.*, 2006).

1.6 Intracellular trafficking

NS3 associates with cellular membrane components through its hydrophobic domains and can interact with both viral and cellular proteins thereby facilitating viral transport from the site of assembly (viral inclusion bodies) to the plasma membrane where virus progeny are released. NS3 therefore exploits the normal host cell protein trafficking pathways in order to aid in viral migration through the cell. These protein trafficking pathways and proteins that play a role in transport in the cell will now be discussed in more detail.

In a classic mammalian cell, a number of proteins are produced some of which are to remain in the cell cytoplasm to function intracellularly and some which are to be transported to the cell surface to be secreted and perform a specific function outside of the cell. For a non-cytoplasmic protein to reach its specific destination two general processes have to occur. The first is to deliver or target the protein to the membrane of one of the intracellular organelles (ER, mitochondria, chloroplasts, peroxisomes and in some cases the nucleus). Membrane proteins are inserted into the lipid bilayer that surrounds the organelle whilst the water-soluble proteins are translocated across the organelle membrane to the aqueous interior. The second process during protein trafficking involves entry of these proteins from the ER membrane into the cell secretory pathway. The secretory pathway allows transportation of proteins inside small vesicles which bud from the membrane of one organelle and fuse with the membrane of the next organelle until the protein reaches its destination. The organelles involved in the secretory pathway include the Golgi complex, lysosomes as well as the plasma membrane (Lodish *et al.*, 2003).

As the transmembrane proteins travel along the secretory pathway, they sustain their original orientation in the membrane so that the domains that face the cytosol remain in the cytosol and vice versa. Proteins that are to be transported to destinations other than the ER and Golgi, reach the *trans*-Golgi network (TGN). From here the proteins are transported to its final destination through one of three different types of vesicles. The first type of vesicle directly fuses with the plasma membrane and the proteins are released by exocytosis. The second type of vesicle is a secretory vesicle. This type of vesicle remains inside the cell until it receives a signal for exocytosis which then triggers the release of the vesicle contents at the plasma membrane. The third type of vesicle is directed to lysosomes. Lysosomes are organelles which are associated with the degradation of macromolecules (Lodish *et al.*, 2003).

Research over years has brought general agreement that the cellular membrane components are compartmentalised into functional microdomains which aid in cellular processes like cell signalling, cell adhesion and membrane trafficking (Laude, Prior, 2004). These microdomains include lipid rafts. Lipid rafts are sphingolipids and cholesterol molecules that cluster into discrete regions inside the cell membrane. These rafts exist in a more orderly state than the other glycerophospholipid-rich domains found elsewhere in the membrane (Salaun *et al.*, 2004). The presence of numerous signalling proteins and lipid cofactors inside lipid rafts suggest that these domains play a role in signalling cascades within the cell (Brown, London, 1998; Simons, Toomre, 2000). Lipid rafts have also been shown to be involved with protein sorting at various other places in the cell as well. In yeast, GPI-anchored proteins associate with lipid rafts inside the ER where their sorting occurs (Bagnat, Keranen *et al.* 2000). Rafts have been shown to play a role in protein entry into the cell at the plasma membrane, in endosomes and lysosomes, and evidence suggests that HIV budding relies on the association of HIV with rafts in exosomes for viral exit through the exosome release pathway (reviewed in Helms and Zurzolo, 2004; Simons and Gerl, 2010). Cuadras *et al.* (2006) showed how rotavirus particles use lipid rafts to aid in viral exit from the host cell. By silencing the two outer capsid proteins VP4 and VP7 and the non-structural protein NSP4 they found that VP4 directly associates with lipid rafts. NSP4 indirectly contributes to rotavirus association with lipid rafts by blocking the transit of particles in the ER. Inhibition of VP7 only slightly affected the migration of virions into the rafts. It is hypothesized that lipid rafts interact with SNARE proteins (soluble N-ethylmaleimide-sensitive factor attachment protein receptors). SNARE proteins normally function in all intracellular membrane fusion events in eukaryotic cells where SNAREs on one membrane fuse with SNAREs on another membrane. The interaction between SNARE proteins and lipid rafts might suggest a direct role of rafts in the membrane fusion reaction (reviewed by Salaun, 2004).

Another microdomain that has been observed on the plasma membrane has been identified as caveolae. Caveolae are small uncoated invaginations approximately 50-70 nm in diameter on the plasma membrane (reviewed by Laude, 2004). Caveolae are characterized by the presence of the protein caveolin (Kurzchalia, Parton, 1996; Rothberg *et al.*, 1992). Caveolin is synthesized inside the ER and oligomerises inside the Golgi after which it is transported to the cell surface. Caveolae in the plasma membrane arise by the merging of small rafts. Caveolae are biochemically very similar to lipid rafts and can be considered as a specialised sub-type of raft (reviewed by Laude, 2004). Caveolae have been suggested to play roles in a number of trafficking processes which include a general regulatory chaperone role (Okamoto *et al.*, 1998), a role in endocytosis, endothelial transcytosis and regulation of cell-signalling processes and cholesterol transport and homeostasis (Simons, Gerl, 2010; van Deurs *et al.*, 2003). Raft-proteins use either the clathrin-mediated endocytic pathway or the caveolin-mediated pathway. Caveolae which bud from the plasma membrane have been shown to contain SNARE proteins (reviewed by Helms, 2004).

Apart from hijacking the normal cell mechanisms for viral egress, viruses can also interact with the cellular cytoskeleton to establish its movement amongst subcellular sites. This involves viral interaction with the host actin and microtubule transport system and their appropriate motor proteins. These mechanisms for intracellular viral trafficking are, however, more applicable to viruses which replicate inside the nucleus (reviewed by Radtke, 2006).

As previously mentioned rotavirus NSP4 – a potential analogue to AHSV NS3 – can interact with caveolae which is one of the pathways that facilitates viral trafficking to the plasma membrane. One of the aims of our study is directed to the investigation of the intracellular trafficking of AHSV NS3 by selectively blocking different pathways. In order to follow the migration of NS3 through the cell, we will investigate the co-localisation of NS3 with markers for cellular compartments by confocal microscopy.

1.7 Fluorescence and Confocal microscopy

My project forms part of a research programme in which the main objective is to understand the role of AHSV proteins in virulence, pathogenicity and disease. The focus of much of this research has rested upon studying the role of NS3/A of AHSV since it is proposed that these proteins are involved in pathogenicity and viral release. To date, very little is known about the association of AHSV NS3 proteins with cellular components and other cellular proteins in infected cells. It is postulated that NS3 associates with cellular components through its hydrophobic domains and is trafficked via the normal cellular vesicular pathway for membrane proteins. NS3 can interact with both cellular and viral proteins and possibly mediates the transport of mature virions from the site of assembly in the cytoplasm to the site of viral egress at the plasma membrane. In BTV there are subsequent differences in the mechanisms of viral release depending on the host cell type. In mammalian cells progeny viruses are released mainly through cell lysis whilst in insect cells viruses exit through budding. In both systems NS3 is suggested to mediate viral release. In this study fourteen different NS3/A constructs will be used in attempt to answer some of the questions regarding the intracellular distribution and trafficking of AHSV in infected cells. Mutation analysis will be performed using six of the fourteen NS3 constructs. These constructs have been made in order to establish the function or contribution of each of the highly conserved domains to the trafficking and localisation of the NS3 protein. The remaining eight constructs contain an eGFP tag on either the N- or C-terminal domain of the NS3 protein. The localisation of these constructs will be compared to immunolabeled NS3 in order to determine if eGFP could be used as a reporter molecule for studying localisation and trafficking of AHSV NS3.

Green fluorescent protein (GFP) is commonly used as reporter molecules for studying intracellular localisation and trafficking of proteins in living systems (Lippencott-Schwartz, Smith, 1997). The

27kDa GFP was first isolated in 1994 from the jellyfish *Aequorea victoria* (Chalfie *et al.*, 1994). GFP emits green light when exposed to blue or UV light and has been found to be useful as a reporter molecule in both insect and mammalian cells (Chalfie *et al.*, 1994). The attractive advantage of the GFP is that protein expression can be directly assayed without prior fixation or treatment and it doesn't require any additional substrates or co-factors. In 1996, Zhang *et al.*, produced the enhanced green fluorescent protein (eGFP) by inducing chromophore mutations which resulted in a 35-fold increase in fluorescence compared to the original protein (Zhang *et al.*, 1996). In this study we will be comparing eight eGFP-tagged NS3 proteins to immunolabelled proteins by means of confocal microscopy in order to establish whether the chimeric proteins localise differently to untagged proteins. This should give an indication of the influence (if any) that eGFP might have on proteins.

Over the past 10 years the Laser Scanning Confocal Microscope (LSCM) has developed to become the tool of choice in studying the localisation and trafficking of proteins inside host cells. The major advantage of the LSCM is that it allows the user to visualize deep into the cells with high resolution. This is based on the principle that all of the out-of-focus light is removed from the image by a suitably positioned pinhole. The sample can then be scanned at different focal planes and the obtained data can be converted to a 3-D representation of the sample. The main drawback of the LSCM used to be the need for a suitable reporter molecule however the development of cellular and protein markers, probes and antibodies for both immunolabeling and live cell imaging have expanded to the extent that general organelle markers are readily commercially available.

Various studies that aimed to resolve questions regarding the intracellular localisation of the non-structural protein NS3 of BTV to specific cellular compartments or components (Bansal *et al.*, 1998; Han, Harty, 2004) or to study the colocalisation of this protein with other cellular proteins, (Wirblich *et al.*, 2006) have used confocal imaging. Through the use of confocal imaging, Wirblich *et al.* (2006) found that BTV NS3 is expressed at the plasma membrane as well as other intracellular membranes in HeLa cells. They also found that the Tsg101 protein (a component of the ESCRT-1 complex which plays a role in intracellular viral trafficking) localises to the same intracellular membranes as the BTV NS3 protein. Based on the colocalisation, the authors then confirmed the interaction of these proteins, through molecular techniques. Han and Harty (2004) showed through specific staining of the ER and Golgi, and immunofluorescence of mutant constructs of the NS3/A proteins that the wild-type BTV NS3/A proteins colocalise with intracellular actin as well as at the Golgi apparatus and in the ER. The mutant constructs however showed no localisation to the Golgi or the plasma membrane. By comparing the localisation of the mutant constructs to that of the wild-type NS3/A proteins, the authors were able to identify those regions on the proteins that are responsible for the specific localisation of the protein within the cells.

Bansal *et al.* (1998) studied the localisation of the BTV NS3/A proteins found that these proteins are processed and glycosylated in the ER and Golgi apparatus.

Confocal imaging has also been applied to study the localisation and trafficking of rotavirus NSP4. The rotavirus NSP4 protein has been found to localise to the ER during the early stages of viral infection whilst NSP4 has also been observed associated with cytoplasmic vesicles (Berkova *et al.*, 2006). The interaction of NSP4 with caveolae and other host cellular proteins needed for trafficking to the plasma membrane have also been studied with cellular protein markers with the use of confocal microscopy (Storey *et al.*, 2007). Recently, confocal microscopy was utilized to study the viroporin characteristics of NSP4 (Hyser *et al.*, 2010).

Preliminary microscopic analysis of the intracellular localisation of AHSV NS3 showed that it had a perinuclear and plasma membrane distribution in AHSV Vero infected cells and a similar distribution when expressed individually in Sf9 insect cells from a recombinant baculovirus (Van Staden *et al.*, 1995). This would correlate with a role for NS3 in membrane based viral trafficking. Recent baculovirus expression of NS3 as a green fluorescent protein (GFP-tagged protein) showed localisation to the nuclear and the plasma membranes. It is not clear whether this distribution of NS3 differed from non-eGFP NS3, and if the distribution was influenced by the eGFP tag (Hatherell, 2007). No comparative studies have ever been done between the distribution of NS3/A in AHSV infected mammalian and insect cells. We will also aim to address the possible differences in localisation of NS3 in insect and mammalian cells as well as attempt to determine the colocalisation of NS3 with specific cellular organelles (ER and Golgi).

Since NS3 is hypothesized to exploit the host cell secretory pathway for migration and release we are also interested in NS3 colocalisation with specific cellular compartments and trafficking proteins. As previously mentioned, there are countless commercially available kits, probes, live cell dyes and antibodies for immunolabeling of specific organelles. The choice of the organelle markers (ER and Golgi) for use in our study will mainly rest upon the application of the marker to more than one cell type (insect and mammalian).

1.8 AIMS OF THIS STUDY

The non-structural AHSV proteins, NS3 and NS3A have been shown to be membrane associated and have been proposed to play a role in pathogenicity and viral release. Individual expression of these proteins has cytotoxic effects on host cells by modifying membrane permeability which leads to cell death (viroporin activity). BTV NS3 associates with the cellular organelle membranes through hydrophobic domains on the protein and it have been suggested that NS3/A exploits the normal vesicular pathway for trafficking. In the case of BTV, NS3/A move via the ER to the Golgi to the plasma membrane and through interaction with viral proteins and host cell proteins, aid in viral release. Viral particles are mainly released through cell lysis in mammalian cells and viral budding in insect cells and in both these systems NS3 has been associated with viral release.

Preliminary microscopic analysis of the intracellular localisation of NS3 has shown that this protein has a perinuclear and plasma membrane distribution in AHSV infected cells and when expressed individually from a recombinant baculovirus. This correlates with the prediction that NS3 is associated with viral trafficking. Recent expression of NS3 green fluorescent tagged proteins (GFP) showed localisation to the nuclear membrane and very little NS3 was present at the plasma membrane. It is not clear whether this distribution of NS3 is due to the GFP tag or as a result of low microscopic resolution. No comparative studies have ever been done between the distribution of NS3/A in AHSV infected mammalian and insect cells.

The aim of this study is to look at the intracellular distribution and trafficking of AHSV NS3, and to relate this to its role in AHSV transport and release in mammalian and insect cells. In order to achieve this we will:

1. Compare the intracellular distribution of wild-type and mutant NS3 proteins expressed in Sf9 (*Spodoptera frugiperda*) cells as native or as eGFP labelled proteins.
2. Compare the intracellular distribution of NS3 in AHSV infected mammalian and insect cell lines.
3. Investigate the colocalisation of NS3 with markers for cellular compartments by confocal microscopy.
4. Investigate the intracellular trafficking of NS3 by using selective chemical blocking of different trafficking pathways.

Chapter 2:

Comparing the localisation and trafficking of wild-type and mutant NS3 proteins in insect and mammalian cells

2.1 INTRODUCTION

AHSV infection has been shown to cause major damage to mammalian host cells while insect host cells are rarely affected (Van Staden, Stoltz *et al.* 1995; Van Niekerk, Smit *et al.* 2001). These findings result from different release mechanisms depending on the host cell type and therefore imply a possible difference in the trafficking of viral particles from the site of assembly to the plasma membrane, possibly also cell type dependent. AHSV non-structural protein NS3 has been associated with pathogenicity and cytotoxicity in both insect and mammalian cells (Van Staden *et al.*, 1995). Early electron microscopy revealed that NS3 localises to the plasma membrane where viruses are released either through cell lysis or by means of viral budding. It has been found that viral budding is dependent on the presence of specific conserved sequences or motifs, called late domains (L-domains). L-domains interact with the host ESCRT proteins to enter the host ESCRT pathway in order to establish trafficking to the plasma membrane and facilitate budding (Licata *et al.*, 2003, Liu *et al.*, 2010, Martin-Serrano *et al.*, 2004, Martin-Serrano *et al.*, 2003, Zhadina and Bieniasz, 2010). Another conserved region found amongst many viruses is a dileucine motif on the C-terminal domains of proteins. This sequence is a trafficking sorting signal for migration from the trans-Golgi network to the plasma membrane (Gonzalez, Rodriguez-Boulan, 2009).

A number of highly conserved regions have been identified in AHSV NS3. These include a proline rich region (L-domain) (aa 22 - 34), a highly conserved region (aa 43 – 92), two hydrophobic membrane spanning domains (aa residues 116-137 and 154-176 respectively) and a putative dileucine motif on the C-terminal of NS3. The two hydrophobic domains are inserted into the membrane in such a manner that both the N- and C-terminal domains of the NS3 protein projects into the cell cytosol (Van Staden *et al.*, 1998) (Figure 5). Because of its topographic distribution in the membrane it is proposed that the N-terminal of NS3 interacts with host cellular proteins whilst the C-terminal of NS3 interacts with newly formed virions in order to establish trafficking to the plasma membrane.

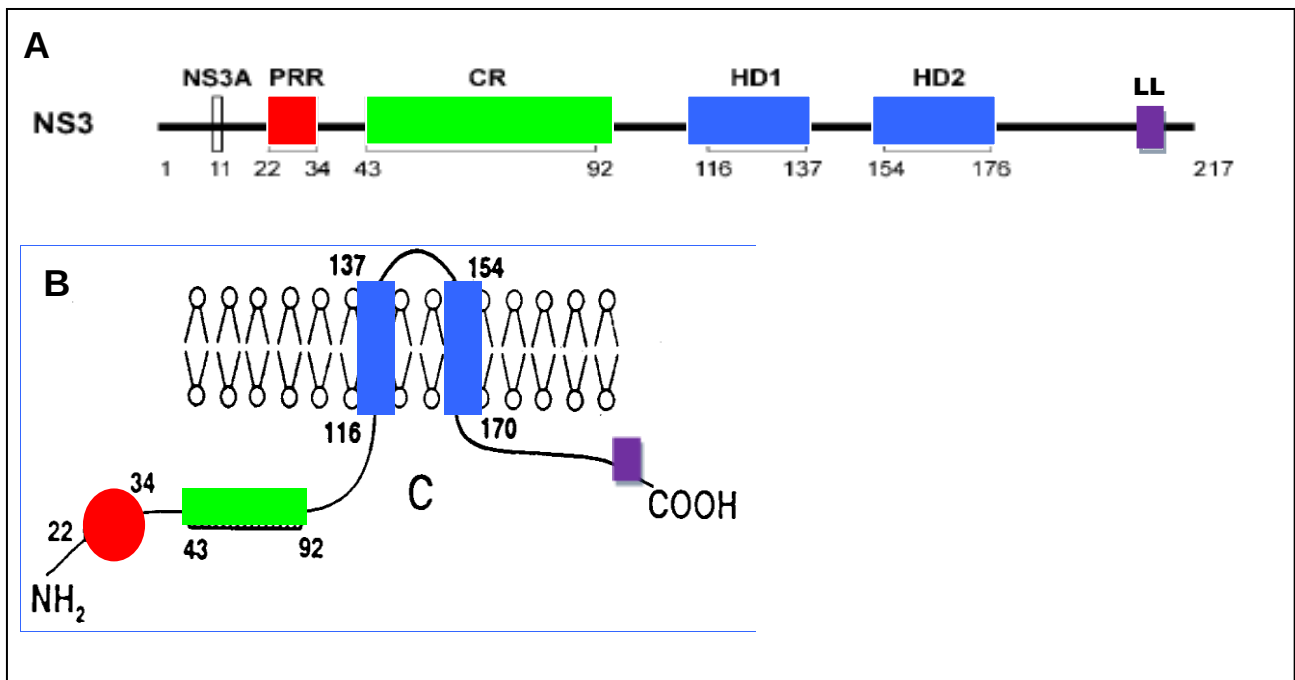


Figure 5. A schematic diagram of the NS3 AHSV proteins' conserved regions (A) and a proposed topographic diagram of NS3 inserted into the lipid bilayer (B). The proline-rich region is shown in red, the conserved region in green, the two membrane spanning domains in blue and the dileucine motif in purple (modified from Van Staden *et al.* 1995).

Previous studies [Hatherell (2007), Smit (1999), Van der Sluis (2008), Van de Merwe (2010)] have used mutational approaches in order to study the cytotoxicity and functionality of the conserved regions of AHSV-3 NS3 in insect cells. In these approaches, NS3 and the mutant variants were all cloned as recombinant baculoviruses for expression in Sf9 insect cells. One study also considered the expression of NS3 as an eGFP fusion protein. However none of these studies have investigated the subcellular localisation of the NS3 proteins extensively.

For this study, we had fourteen recombinant baculovirus NS3 constructs available to us (see Results section, Table 5 for details). These proteins included wild-type NS3, NS3 with each of the conserved regions mutated as well as eight eGFP-tagged NS3 proteins. In this study we compared the intracellular localisation of each of the mutant proteins to the wild-type NS3 protein as well as comparing the localisation of eGFP-tagged and untagged proteins in order to establish whether the tag has any effect on protein localisation. To ascertain whether there are different trafficking mechanisms of NS3 proteins in insect cells as opposed to mammalian cells, we compared the localisation of NS3 proteins in recombinant baculovirus infected Sf9 insect cells to AHSV infected Vero cells. In a separate experiment, both cell types were also treated with Brefeldin A, a chemical inhibitor which disrupts trafficking from the endoplasmic reticulum to the Golgi apparatus. This was done to determine whether the NS3 protein uses the cellular secretory pathway for membrane bound proteins. In this cellular pathway, proteins are inserted into the lipid bilayer of a membranous organelle (vesicles or ER) and buds from one organelle to the next until the protein

reaches its target destination. Analysis of infected cells was mainly done with confocal microscopy in an attempt to get a clearer understanding of protein localisation and trafficking.

2.2 MATERIALS AND METHODS

2.2.1 Plasmids

Listed below (Table 2) are all the plasmids that were available and used during the course of this study.

Table 2: Plasmids:

Plasmid	Protein encoded	Additional notes	Produced by
pUC-S10	NS3	Full-length AHSV-3 Seg-10 inserted into a BamHI site of pUC-13 (Amp ^R)	Dr V van Staden*
pBlueScribe-NS3A	NS3A	Amp ^R	C Smit*
pFastbac-HD1	NS3-HD1	Gent ^R Amp ^R	C Smit*
pFastbac-HD2	NS3-HD2	Gent ^R Amp ^R	C Smit*
pFastbac-PRO	NS3-PRO	Gent ^R Amp ^R	C Smit*
pFastbac-CR	NS3-CR	Gent ^R Amp ^R	C Smit*
pCR®8/GW/TOPO®	None	Vector supplied with pCR®8/GW/TOPO® TA cloning® kit to serve as entry vector for the Gateway cloning system (Spec ^R)	Invitrogen
pDEST8	None	Vector supplied with pCR®8/GW/TOPO® TA cloning® kit to serve as destination vector for the Gateway cloning system (Gent ^R Amp ^R)	Invitrogen

*Department of Genetics, University of Pretoria

2.2.2 Recombinant baculoviruses

Listed below are the recombinant baculoviruses that were used during the course of this study.

Table 3: Baculoviruses:

Virus	Protein	Additional notes	Produced by
Bac-NS3	NS3	Full-length (aa 1 - 217) wild-type AHSV-3 NS3 protein	C Smit*
Bac-NS3A	NS3A	NS3 deletion mutant lacking the first 11 N-terminal amino acids of NS3	C Smit*
Bac-NS3-PRO	NS3-PRO	NS3 substitution mutant with amino acids 25 - 27 (PPP substituted with DVR)	C Smit*
Bac-NS3-CR	NS3-CR	NS3 substitution mutant with amino acids 77 - 82 (AEARLD substituted to VDLQKE)	C Smit*
Bac-NS3-HD1	NS3-HD1	NS3 substitution mutant with amino acids 124 -128 (VTMAT substituted with RTRDK)	C Smit*
Bac-NS3-HD2	NS3-HD2	NS3 substitution mutant with amino acids 165 - 169 (MLLA substituted with KRDV)	C Smit*
Bac-eGFP	eGFP	Full-length wild-type eGFP protein	M Victor*
Bac-NS3-eGFP	NS3-eGFP	Full-length (aa 1 - 217) wild-type AHSV-3 NS3 protein with eGFP on the C-terminus	T Hatherell*
Bac-eGFP-NS3	eGFP-NS3	Full-length (aa 1 - 217) wild-type AHSV-3 NS3 protein with eGFP on the N-terminal	R van der Sluis*
Bac-NS3A-eGFP	NS3A-eGFP	NS3 deletion mutant lacking the first 11 N-terminus amino acids of NS3 with eGFP on the C-terminus	T Hatherell*
Bac-NS3ΔC-eGFP	NS3ΔC-eGFP	NS3 with a 14 amino acid C-terminus deletion (which include the dileucine motif) with eGFP (C-terminus)	T Hatherell*
Bac-N11-eGFP	N11-eGFP	The first 11 N-terminus amino acids of NS3 with eGFP on the C-terminus	T Hatherell*
Bac-HD1-eGFP	HD1-eGFP	NS3-HD1 with eGFP on the C-terminus	T Hatherell*
Bac-HD2-eGFP	HD2-eGFP	NS3-HD2 with eGFP on the C-terminus	T Hatherell*

*Department of Genetics, University of Pretoria

2.2.3 Antisera

Listed below are the antibodies that were used during the course of this study.

Table 4: Antibodies:

Antibody	Specificity and dilution	Source
Anti-18aa-NS3 AHSV-3	Antibodies raised in rabbits against an 18 amino acid conserved region on the C-terminal domain of AHSV-3 NS3 (CISDKIQEEISRTKRDI A) (1:100*)	Genscript
Anti-eGFP	Antibodies raised in rabbits against the N-terminal domain of eGFP (1:5 000*)	Sigma (Cat no G1544)
Anti-rabbit Alexa Fluor® 488	Fluorescently labelled antibodies (green) goat anti-rabbit IgG (1:250*)	Invitrogen (Cat no A11008)
Anti-mouse Alexa Fluor® 594	Fluorescently labelled antibodies (red) goat anti-mouse IgG (1:250*)	Invitrogen (Cat no A11032)
golgin-97 (human) mouse IgG (anti-Golgi)	Antibodies raised in mice directed against a 97 kDa peripheral Golgi membrane protein (1:100*)	Invitrogen (Cat no A21270)
CellLight™ ER-RFP *BacMam 2.0*	Fluorescent protein based reagent directed against the endoplasmic reticulum targeting sequence of calreticulin	Invitrogen (Cat no C10591)
Anti-Calnexin	Monoclonal antibody that binds to ER protein calnexin (1:100*)	BD Biosciences
Anti-β-Tubulin	Monoclonal antibody that binds to the C-terminal domain of β-tubulin which makes up microtubules (1:100*)	Sigma (Cat no T5201)
Anti-vimentin	Monoclonal antibody that binds to vimentin protein (1:100*)	Sigma (Cat no V6389)

*Diluted in 1% blocking solution.

2.2.4 Cells

Bacterial strains used were *Escherichia coli* XL1Blue cells, and DH5α cells or Top10 cells (pCR®8/GW/TOPO® TA cloning® kit, Invitrogen) or DH10bac cells (BAC-to-BAC™ expression system. Cells (with the exception of Top10 cells) were grown in a 3 ml liquid LB-Broth (1% Bacto-Tryptone (w/v), 0.5% Bacto-Yeast extract (w/v), 1% NaCl (w/v) pH 7.4) at 37°C containing the appropriate antibiotics. Bacterial cultures were stored in the presence of 15% glycerol at -70°C.

Spodoptera frugiperda (Sf9) insect cells were utilized for all baculovirus expression work. Sf9 cells were cultured and maintained in spinner flasks at 27°C in TC100 medium [Highveld Biological (PTY) Ltd] containing 10% foetal calf serum, 1% pluronic (Sigma) and antibiotics (Penicillin – 100 U/ml, Streptomycin – 0.1 mg/ml and fungizone – 2.4 µg/ml). Cells were counted using a Bright-Line haemocytometer (Hausser scientific) under a Nikon TMS E100.25 light microscope.

Vero, BSR and baby hamster kidney (BHK) cells were cultured and maintained in 75cm² flasks in minimal essential medium with nonessential amino acids (Highveld Biological) supplemented with 5% foetal calf serum (FCS) and antibiotics (fungizone - 2.5 µg/ml, penicillin - 100 U/ml and streptomycin - 100 µg/ml (Highveld Biological)). Mammalian cells were maintained at 37°C in the presence of 5% CO₂.

2.2.5 Polymerase Chain Reaction

PCR reactions were set up as follows: 50 µl reactions containing approximately 50 ng of pUC-S10 template DNA, 100 pmol of each of the NS3ABamF forward (5'-GCGGATCCATGATGCATAATGGAAATC-3') and NS3pEco reverse (5'-CGGAATTCGTAAGTCGTTATCCCGG-3') oligonucleotide primers, 5 µl of 10 x ExTaq polymerase buffer (500mM KCl, 100mM Tris.HCl, 1% Triton X-100) (TaKaRa), 4 µl of 25 mM MgCl₂ solution, 10 µl of a 1 mM dNTP mixture (TaKaRa) and 5 U of ExTaq polymerase (TaKaRa). The PCR reactions were performed in a Perkin-Elmer 9600 Gene Amp PCR system under the following conditions: An initial template denaturation step occurred at 95°C for 5 min, this was followed by denaturation for 30 s at 95°C, a primer annealing step for 45 s at 52°C. Primer extension and polymerization occurred at 72°C for 90 s. After 30 cycles of amplification final elongation occurred at 72°C for 15 min.

Amplicons were analysed and verified by 1% agarose gel electrophoresis.

2.2.6 Agarose gel electrophoresis

Agarose gel electrophoresis was used to analyse and separate DNA fragments. Products of interest were separated on a gel consisting of 1% (w/v) agarose dissolved in 1 x TAE buffer (0.04 M Tris-acetate, 1mM EDTA, pH 8.5). 1% Ethidium bromide was added to the gel to enable visualisation of DNA bands under UV light. Electrophoresis occurred in electrophoresis tanks where gels were submerged in 1 x TAE buffer for approximately 1 hour at 100 V constant current. Gels were visualised on a UV transilluminator and the DNA band sizes were estimated by comparison with standard molecular weight size markers which included the O'RangeRuler™ 100bp+500bp DNA ladder (Fermentas).

2.2.7 Plasmid DNA isolation

Plasmid isolations were performed using the alkaline-lysis method according to the protocol described by Sambrook and Russell (2001). Single bacterial colonies were picked from an agar plate using sterile toothpicks. Each colony was then transferred to a 3 ml volume of LB-Broth (1%

Bacto-Tryptone (w/v), 0.5% Bacto-Yeast extract (w/v), 1% NaCl (w/v) pH 7.4) containing the appropriate antibiotics. Cultures were grown overnight at 37°C with shaking until they reach saturation. A 1.5 ml volume of the overnight culture was used to collect cells by means of centrifugation at 13 000 rpm for 5 min. Cell pellets were resuspended in 300 µl of ice-cold lysis buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris) and incubated on ice for 5 min. The addition of 300 µl of 0.2 M NaOH and 1% SDS with 5 min incubation on ice was to denature proteins and double stranded DNA (dsDNA) and to solubilise the bacterial membrane to release the plasmid DNA into the supernatant. For the precipitation of the dsDNA and high molecular weight RNA, 300 µl NaAc (pH 4.8) was added and tubes were incubated on ice for 5 min. Samples were subjected to centrifugation at 13 000 rpm for 10 min after which the supernatant was transferred to a clean Eppendorf tube and 500 µl of Propan-2-ol (isopropanol) was added to precipitate plasmid DNA. After a 20 min incubation on ice, DNA was pelleted by centrifugation at 13 000 rpm for 10 min. The pellet was washed twice with 70% ethanol (EtOH) and allowed to air dry. The pellet was resuspended in 40 µl of ultra-high quality water (UHQ) and visualised by 1% agarose gel electrophoresis as described previously.

2.2.8 Purification of DNA fragments

PCR products were purified using the commercially available High Pure PCR Product Purification kit (Roche). This kit allows for the selective binding of DNA to pre-packed glass fibres in the filter tubes. The bound DNA is washed with ethanol to remove all contaminants (primers, nucleotides and salts) after which the DNA is eluted in UHQ.

After plasmid DNA fragments were subjected to agarose gel electrophoresis, selected bands were excised from the 1% gel with a razor blade. The gel slice was incubated with binding buffer (3 M guanidine-thiocyanate, 10 mM Tris-HCl, 5% ethanol (v/v), pH 6.6) at 56°C for 10 minutes. The binding buffer lowers the melting temperature of agarose as well as facilitates the binding of the dsDNA to the glass fibres. Once the gel was completely dissolved, isopropanol was added to the mixture and vortexed thoroughly. The mixture was transferred to a High Pure filter tube and centrifuged for 1 min at 13 000 rpm. The bound DNA was washed twice with wash buffer (20 mM NaCl, 2 mM Tris-HCl, pH 7.5) after which the DNA was eluted with UHQ.

2.2.9 Restriction endonuclease digestions

Restriction endonuclease digestions were performed in the appropriate buffers (Roche) (according to the manufacturer's instructions). Plasmid digestions were carried out in a total volume of 30 µl. Each reaction contained approximately 1 µg of DNA, 3 µl of the appropriate 10 x RE buffer, 8 U of the appropriate RE enzyme. Reactions were incubated overnight at 37°C and the DNA was

analysed by electrophoresis in 2% agarose gels. Band sizes were estimated by comparison with molecular weight markers of known size which were either O' GeneRuler™ 100 bp ladder (Fermentas) or O' RangeRuler™ 100bp+500bp ladder (Fermentas).

2.2.10 Ligation of DNA fragments

A standard ligation protocol was used to perform construct ligations. Reactions were carried out in a total volume of 20 µl. Each reaction contained 2 µl 10 x ligase buffer (66 mM Tris-HCl, 5 mM MgCl₂, 1 mM DTT, 1 mM ATP, pH 7.5), 2 U of T4 DNA ligase and DNA fragments in an insert: vector ratio of 2:1. Reactions were incubated overnight at 16°C.

2.2.11 Preparation of competent *E. coli* cells

Electrocompetent and chemically competent cells were used for transformations.

Cells were prepared by picking a single bacterial colony from an agar plate with a sterile toothpick and transferring it to a 3 ml culture of LB-Broth containing antibiotics (if required). The sample was incubated overnight at 37°C with moderate shaking. A 2 ml sample of the overnight culture was transferred to 250 ml pre-warmed LB-Broth containing antibiotics (if required). The culture was allowed to grow to log phase (OD₆₀₀ of 0.5) at 37°C with shaking.

For electrocompetent cells, cells were incubated on ice for 20 min and collected by centrifugation at 5000 rpm for 10 min at 4°C. After the supernatant fluid was discarded, cell pellets were washed by mixing (swirling) with 5 ml sterile ice cold ddH₂O. After the addition of another 120 ml of ice cold ddH₂O, cells were centrifuged at 5000 rpm for 10 min at 4°C. The supernatant fluid was poured off immediately and the pellet was resuspended by swirling in the remaining liquid. A second wash step was performed by adding another 150 ml of ice-cold ddH₂O to the cells after which they were pelleted by centrifugation at 5000 rpm for 10 min at 4°C. The supernatant fluid was immediately discarded and cells were resuspended in 10% glycerol. The cells were incubated on ice for 60 min after which they were centrifuged at 5000 rpm for 10 min at 4°C. The supernatant fluid was discarded and final pellets were resuspended in 1 ml 10 % glycerol. Cells were aliquoted into sterile Eppendorf tubes (50 µl per tube) and stored at -70°C.

For chemically competent cells, cells were collected by centrifugation at 5000 rpm for 10 min at 4°C. The supernatant fluid was discarded and the pellet was resuspended in 10 ml of 50 mM CaCl₂. After cells were collected by centrifugation (5000 rpm for 10 min at 4°C) the supernatant fluid was discarded and the pellet was resuspended in 1/20 of the original volume of 50 mM CaCl₂. Competent cells were stored at -70°C.

2.2.12 Transformation of recombinant DNA into competent cells

Competent bacterial cells were transformed using either electroporation (DH10Bac™ cells) or the heat shock method.

For electroporation, a 20 µl ligation mixture containing between 10 – 50 ng of DNA was mixed with electrocompetent bacterial cells by gently swirling. The cell/ligation mixture was transferred to a pre-cooled electroporation cuvette (Biorad Gene Pulser cuvette) and pulsed at 2000 V. Directly after the electroporation 1ml of LB-Broth was added to the cells which was transferred to a test tube and incubated for 60 min at 37°C with shaking. A dilution series of cells were plated onto agar plates containing the relevant antibiotics and incubated overnight at 37°C.

For the heat shock method 100 µl of chemically competent bacterial cells was mixed with 10 µl of the appropriate ligation mixture and incubated on ice for 30 min to allow the DNA to bind to the cell membranes. Cells were permeabilised by heat shock at 42°C for 90 s followed by a 2 min incubation on ice. Cells were mixed with 900 µl of pre-warmed LB-Broth and incubated at 37°C with shaking for 60 min. A dilution series of cells were plated out onto agar plates containing the relevant antibiotics and plates were incubated overnight at 37°C.

2.2.13 Sequencing

Plasmid sequencing was carried out by means of dye-terminator cycle sequencing using the ABI Prism™ 310 Genetic analyser on highly purified plasmid DNA. Reactions were performed using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit, version 3.0 (Perkin Elmer Applied Biosciences) according to the manufacturer's protocol. In short: 10 µl reactions were set up containing 200 ng of plasmid DNA, 3.2 pmol of either the M13F (5'-TGTAACGACGGCCAGT-3') forward primer or the M13R (5'-CAGGAAACAGCTATGACC-3') reverse primer, 1 µl 5 x BigDye sequencing buffer and 2 µl of BigDye. Sequencing reactions were carried out in a Perkin-Elmer 9600 Gene Amp PCR system under the following conditions: template denaturation at 96°C for 10 s, primer annealing at 50°C for 10 s and extension at 60°C for 4 min. After 25 cycles the reaction was cooled down to 4°C.

The sequencing reactions were cleaned and precipitated as follows: Each sample was made up to a volume of 20 µl with UHQ. After the addition of 50 µl of ice cold 95% EtOH the sample was centrifuged at 13 000 rpm for 30 min. The supernatant was discarded and 250 µl 70% EtOH was added to the pellet. The sample was centrifuged at 13 000 rpm for 10 min and the supernatant was discarded. The pellet was left to air dry overnight.

Sequences were analysed using the Vector NTI version 9.0 program and the Primer Design 4 programmes.

2.2.14 TOPO cloning

The pCR®8/GW/TOPO® TA cloning® kit was used to generate a NS3A entry clone according to the manufacturers protocol. In short: 4 µl of freshly prepared PCR product, 1 µl salt solution and 1 µl pCR®8/GW/TOPO® vector was mixed and incubated at room temperature for 5 min. A volume of 2 µl of the vector solution was incubated with chemically competent Oneshot Top10 cells (Invitrogen) on ice for 30 minutes to allow binding of the vector to the cell membrane. The cells were treated by heat shock at 42°C for 30 s and immediately transferred to ice. The cells were mixed with 250 µl pre-warmed S.O.C medium (Invitrogen) and incubated at 37°C with shaking for 1 hour. Volumes between 10 and 50 µl were plated out on LB agar plates containing 100 µg/ml spectinomycin.

2.2.15 LR Recombination

For the LR recombination reaction, 100 ng of the TOPO entry clone was mixed with 150 ng of the pDest8 destination vector and 10 U clonase II enzyme at 25°C overnight. To denature all unreacted proteins, 5 U proteinase K was added and incubated at 37°C for 10 min. The reaction was placed on ice and directly transformed in chemically competent DH5α cells by heat shock treatment. Cells were plated out on LB agar plates containing 100 µg/ml ampicillin.

2.2.16 Generation of recombinant bacmids

Recombinant baculoviruses were prepared according to the GIBRO BRL Bac-to-Bac™ baculovirus expression (Invitrogen Life Technologies) manual. The recombinant vectors were mixed with 100 µl of electrocompetent DH10Bac™ cells and incubated on ice. The recombinant vector was transformed into the DH10Bac™ cells by means of electroporation as described in section 2.2.12. DH10Bac™ cells were grown on plates containing 7 µg/ml gentamycin, 10 µg/ml tetracyclin, 50 µg/ml kanamycin, 100 µg/ml XGal and 40 µg/ml IPTG. The bacmid DNA contains a LacZα which enables α-complementation of the β-galactosidase gene for blue/white screening. Cells containing the recombinant DNA were characterised by a white phenotype. After 48 hours of incubation at 37°C white colonies were restreaked on similar agar plates to confirm the white phenotype.

2.2.17 Isolation of recombinant bacmid DNA

Transformed DH10Bac™ cells were grown in 3 ml LB Broth (7 µg/ml gentamycin, 10 µg/ml tetracyclin, 50 µg/ml kanamycin) at 37°C with shaking for 16 hours. Cells were collected by centrifugation at 13 200 rpm for 10 min. The cell pellet was resuspended in 300 µl Solution I (15 mM Tris-HCl pH 8, 10 mM EDTA) and after the addition of 300 µl of Solution II (0.2 N NaCl, 1 % SDS) the cells were incubated at room temperature for 5 min. Potassium acetate (300 µl) (3M, pH 5.5) was added and the cells were incubated on ice for 10 min. After centrifugation at 13 200 rpm for 10 min the supernatant fluid of the two samples were transferred to a new eppendorf tube containing 800 µl isopropanol. The sample was gently mixed and incubated on ice for 10 min and centrifuged at 13 200 rpm for 10 minutes. After three washes with 70% ethanol, the pellet was air dried and resuspended in 40 µl UHQ.

2.2.18 Transfections of Sf9 insect cells with bacmid DNA and amplification of viruses

Spodoptera frugiperda (Sf9) insect cells, obtained from American Type Cell Collection were seeded in mono-layers on 6-well plates at a concentration of 1×10^6 cells/well and incubated at 27°C for 1 hour to allow the cells to attach. For each well of the 6-well plate, 6 µl of CELLFECTIN™ (Invitrogen Life Technologies) was mixed with 100 µl non-supplemented TC100 medium in an Eppendorf tube. Separately, 6 µl of isolated bacmid DNA was mixed with 100 µl non-supplemented TC100 medium in a second eppendorf tube. The contents of the two tubes were gently mixed and incubated at room temperature for 45 minutes and increased to a final volume of 1 ml with non-supplemented TC100 medium. The Sf9 cells were rinsed twice with serum-free TC100 medium and overlaid with the DNA lipid mixture. Cells were incubated at 27°C for 5 hours to allow transfection to occur. After this period, the lipid DNA mixture was removed and the cells were overlaid with complete TC100 medium and incubated at 27°C for 4 days after which the cells were harvested and the supernatant was used to infect 75 cm² flasks containing 1×10^7 cells in order to amplify the baculovirus. After two rounds of amplification the supernatants containing the recombinant baculoviruses were harvested, filter sterilized and stored at 4°C. The titre of the baculovirus stock was then determined.

2.2.19 Titration of virus stocks

Viruses titres were determined as described by the BAC-to-BAC™ Baculovirus expression system manual (Invitrogen Life Technologies). Sf9 cells were seeded on 6-well plates at 8.5×10^5 cells/well (55% confluency) and incubated at 27°C for 1 – 2 hours to allow the cells to attach. A ten-fold dilution series of the amplified baculovirus stocks was prepared using supplemented

TC100 medium. A 500 µl of sample of the appropriate dilution was added to a well and incubated at room temperature for 1 hour. A 2% (w/v) Agarose solution, Type VII (Sigma) was prepared in UHQ, boiled and cooled down to approximately 50°C from which a 1% working stock was prepared by adding an equal volume of pre-warmed (38°C) double strength Grace's medium (Highveld Biological). The dilutions were gently aspirated and replaced with the 1% agarose/medium mixture (2 ml/well) and incubated at 27°C for 7 days until the plaques were visible. The cells were stained with 500 µl/well 0.1% Thiazolyl blue Tetrazodium Bromide (Sigma) dissolved in 1 x PBS and were then incubated at 27°C for 24 hours. The number of visible plaques were counted and used to calculate the viral titre as follows: Titre (pfu/ml) = 2 x number of plaques x dilution factor.

2.2.20 Baculovirus infection of Sf9 cells for expression analysis

Sf9 insect cell seeded at 1×10^6 cells/ 35mm well were infected with the recombinant baculovirus of interest at a multiplicity of infection (MOI) of 5 plaque forming units/ml (pfu/ml). Plates were incubated at 27°C for 48 hours. Cells were harvested and collected by centrifugation at 4000 rpm for 10 min at 4°C. The cell pellets were washed twice with sterilized 1 x PBS buffer, resuspended in 1 x PBS buffer and stored at -20°C for analysis by SDS-PAGE and Western blot.

2.2.21 SDS-PAGE

Proteins were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions as described (Sambrook, Russel, 2001). Proteins were loaded and separated on a discontinuous stack according to size differences. A 15% separating gel (15% polyacrylamide, 0.375 M Tris-HCl pH 8.8, 0.1% SDS, 0.008% TEMED, 0.08% ammonium persulphate) was overlaid with a 5% stacking gel (5% polyacrylamide, 0.125 M Tris-HCl pH 6.8, 0.1% SDS, 0.008% TEMED, 0.08% ammonium persulphate). An equal volume of protein sample was mixed with 2 x protein solvent buffer (PSB; 0.125M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercapto-ethanol, 0.005% Bromophenol blue) and heated to 95°C for 2 – 5 min and then loaded into the wells on top of the stacking gel. Gels were run in 1 x TGS buffer (0.3% Tris, 1.44% Glycine, 0.1% SDS) at 130 V in a Hoefer™ II SE 250 mini-vertical gel electrophoresis unit (Amersham Biosciences) for approximately 3 hours or until the PageRuler™ Rainbow protein molecular weight marker (Fermentas) had reached the bottom of the gel.

2.2.22 Western blot

After proteins were separated on an SDS-PAGE gel, proteins were transferred to a nitrocellulose membrane (Hybond-C+, Amersham Biosciences) submerged in Transfer buffer (0.025 M Tris, 0.15 M Glycine, 20% Methanol pH 8.8) in an EC 140 miniblott module at 160 mA for 1 hour. The

nitrocellulose membrane was washed once with 1 x PBS for 5 min with gentle agitation to eliminate the methanol. The membrane was incubated in 1% blocking solution (1% fat free milk powder in 1 x PBS) at room temperature for 1 hour with shaking. The membrane was then incubated in primary antibody (diluted in 1% blocking solution – see 2.2.3) overnight at room temperature with gentle shaking. The membrane was subjected to 3 washes of 5 min in Wash buffer (0.05% (v/v) Tween in 1 x PBS). The membrane was incubated in Protein A Peroxidase conjugate (1:10 000 in 1% blocking solution) for 1 hour at room temperature with gentle agitation. The membrane was washed 3 times in Wash buffer for 5 minutes at a time to eliminate the excess antibodies. To develop the protein bands, the membrane was incubated in substrate solution (60 mg 4-chloro-naphtol in 20 ml chilled methanol, 60 µl in 100 ml 1 x PBS) in the dark for 10 min. Once the protein bands were visible the membrane was rinsed with distilled water to stop the reaction.

2.2.23 Subcellular fractionations

Subcellular fractionations were performed according to a protocol adapted and optimised by our laboratory (Beylerveld, 2007; Hatherell, 2007; Meiring, 2009) from that used by Brignati *et al* (2003). Sf9 insect cells (seeded at 1×10^6 cells/35 mm well) were infected with recombinant baculovirus at an MOI of 10 pfu/cell and incubated at 27°C for 48 hours. Cells were harvested and collected by centrifugation at 4000 rpm for 10 min at 4°C. A fraction of these cells was resuspended in 1 x PBS and used subsequently as a control fraction whilst the rest of the cells were washed once with 1 x PBS buffer and resuspended in 300 µl hypotonic buffer (10 mM Tris, 0.2 mM MgCl₂ pH 7.4) containing protease inhibitors (1.0mg/ml Pefabloc (Roche), 0.7 µg/ml Pepstatin (Roche)). The suspension was incubated on ice for 30 min after which cells were mechanically lysed by 6 passages through a 22G needle. Unbroken cells and nuclei were removed by low speed centrifugation (1500 rpm) for 2 min. The supernatant was transferred to a clean Eppendorf tube whilst the pellet was resuspended in 50 µl hypotonic buffer and centrifuged at 1500 rpm for 2 min. The pellet was resuspended in 1 x PBS (labelled N – nuclear fraction). The supernatant was pooled with the initial supernatant which contained all the cellular membranes and soluble proteins. The membranes and all proteins associated with them were pelleted by centrifugation at 13 000 rpm for 1 hour at 4°C. The pellet was resuspended in 1 x PBS and labelled P – particulate fraction. The supernatant fluid was transferred to a clean Eppendorf tube and the soluble proteins were concentrated by vacuum vaporisation. The latter sample was labelled S, since it contained all the soluble proteins. Protein fractions were analysed by SDS-PAGE (2.2.21) and Western blot (2.2.22).

2.2.24 Membrane flotation analysis

For membrane flotation analysis, cells were grown, infected, harvested and treated as described in 2.2.24. After cells were mechanically lysed by passage through a needle, a volume of 250 μ l of the lysed cells was gently mixed with 1340 μ l of 80% sucrose in 1 x NTE buffer (100 mM NaCl, 10 mM Tris, 1 mM EDTA [pH 7.4]) and loaded at the bottom of a 5 ml polyallomer tube (Beckman). This bottom fraction had a final concentration of 72% sucrose. The bottom fraction was overlaid with 2320 μ l of 65% sucrose (in 1 x NTE) and a final layer of 1090 μ l of 10% sucrose (in 1 x NTE) was added on top of the gradient. The gradients were centrifuged at 38 000 rpm for 18 hours at 4°C in a Beckman SW55Ti rotor using a Beckman Coulter Optima™ L-100 XP Ultracentrifuge. Fractions of 200 μ l were collected by means of pipetting from the top of the gradient. The fractions were labelled in reverse from 10 to 1 so that the top fraction corresponded to fraction 10 and the last fraction at the bottom of the tube was labelled 1. The pellet was resuspended in 200 μ l 1 x NTE and was also included in analysis. Non-eGFP linked NS3 proteins were analysed by means of SDS-PAGE and Western blot. eGFP-tagged NS3 proteins were analysed with a fluorometer (Fluoroskan Ascent FL Type 374; Thermolabsystems) using appropriate filter sets (485–495_{Ex}/515–525_{Em}). The measurements were captured using the Ascent Software Version 2.4.2. These values were used to draw line graphs using Microsoft Office Excel 2007.

2.2.25 Confocal microscopy

Sf9 cells were seeded on sterile Esco™ coverslips (22mm x 22mm) in 6-well plates at 1×10^6 cells/35 mm well. Cells were infected with wild-type or recombinant baculoviruses at an MOI of 10 pfu/cell and incubated at 27°C for 24 hours. Vero cells were seeded on sterile Esco™ coverslips (22mm x 22mm) in 6-well plates at 2.5×10^5 cells/35 mm well (quarter of the normal cell density). Vero cells were infected with AHSV-3 (MOI of 5) and incubated at 37°C with CO₂ for 24 hours.

Cells were washed twice with sterile 1 x PBS buffer (5 min per wash). Cells were fixed and rinsed as described below. Different fixation methods were tested: either 50% (v/v) methanol/acetone, or 4% paraformaldehyde (Harlow and Lane, 1999) with or without subsequent treatment with Triton X-100. The latter fixative was only tested on immunolabelled cells since these cells need permeabilisation of the cell membranes to allow antibody penetration.

After cells were washed once with 1 x PBS, cells were fixed with ice-cold 50% (v/v) methanol/acetone for 2 min. The fixative was extracted and replaced by 1 x PBS buffer for 5 min. Alternatively cells were fixed with 4% paraformaldehyde (4% paraformaldehyde dissolved in 1 x PBS) at room temperature for 15 min. Cells were given 3 separate washes in wash buffer (0.5% (v/v) Tween in 1 x PBS) and for immunolabelled samples, cells were subsequently incubated with

Triton X-100 (0.1% Triton X-100 in 1 x PBS) at room temperature for 10 min. Cells were washed once with 1 x PBS for 5 min.

Vero cells and Sf9 cells expressing non-eGFP NS3 proteins, were then incubated in 5% blocking solution (5% milk powder in 1 x PBS) for 30 min at room temperature after fixation. This was followed by incubation in primary antibody (as described in 2.2.3) at 4°C overnight. For colocalisation studies, cells would be incubated with both primary antibodies simultaneously. Cells were subjected to 3 separate wash steps with Wash buffer [0.5% (v/v) Tween in 1 x PBS] to remove the excess antibody. This was followed by incubation in the appropriate secondary antibody/antibodies (as described in 2.2.3) for 1 hour at room temperature. Coverslips were mounted and visualised. All mounted slides were stored in the dark at room temperature.

For nuclear staining, cells were incubated with 10 µg/ml DAPI (4'6'-diamidino-2-phenylindole) for 10 min at room temperature. Coverslips with cells expressing eGFP-tagged NS3 proteins were mounted onto microscope slides using VECTASHIELD mounting medium (Vector Laboratories) to prevent photobleaching. Coverslips were sealed around the edges prevent them from drying out.

Slides were analysed using the Zeiss LSM510 Meta Laser Scanning Confocal Microscope. All images were captured using the Zeiss LSM Image Browser version 4.0.0.157. Alexafluor488™ and eGFP were detected at 488 nm, DAPI was detected at 405 nm and Alexafluor594™ was detected at 543 nm. Colocalisation analysis was performed with ImageJ freeware version 1.43j.

2.2.26 Brefeldin A treatment

Sf9 cells were seeded on sterile Esco™ coverslips (22mm x 22mm) at 1×10^6 per 35 mm of a 6-well plate. Cells were infected with recombinant baculovirus and incubated at 27°C. At 16 hpi, the culture medium was replaced with 2 ml non-supplemented TC-100 medium containing 10µg/ml brefeldin A (BFA; MP Biomedical). This was repeated at 4 hour intervals to prevent depletion of BFA. Cells were processed at 27 hpi.

2.2.27 Co-localisation analysis

All co-localisation analyses were performed using the ImageJ software version 1.43j (<http://rsb.info.nih.gov/ij>). Images were analysed in JPEG format. Firstly the image was split into the different channels. This function automatically converts each image to an 8-bit file. Images that represent the blue channel were discarded since these would not influence or be included in the co-localisation analysis. The images were scaled using the scale bar at the bottom of each image. For each image the threshold values for each channel (red and green) was determined and

specified. All analyses were performed using the red channel as reference channel since we were interested in the co-localisation of the NS3 protein (green channel) with the ER or Golgi (red channel). The region or regions of interest (ROI) was specified using the ROI manager. The number of pixels in each ROI was calculated for both the red and green channels and the co-localisation analysis was performed. The co-localisation image was converted to an 8-bit black and white image. By using the same ROI as in the red and green channels, the number of pixels that represent co-localisation was calculated for each ROI. All data was recorded in Microsoft Office Excel 2003. The percentage co-localisation was calculated by dividing the number of colocalised pixels by the number of pixels in the red channel.

2.3 RESULTS

The aim of this study was to investigate the subcellular localisation and trafficking of AHSV-3 NS3. A number of constructs encoding mutant NS3 proteins had previously been generated. Some of these constructs had been used to generate recombinant bacmids, and a few of these had been used to infect Sf9 cells to generate baculovirus stocks (summarized in Table 5). Before we could start the comparative analysis, the NS3A construct had to be generated, some constructs had to be recombined to bacmids and the outstanding baculoviruses had to be produced. Once all the NS3 mutants could be expressed from recombinant baculoviruses, the investigation of NS3 localisation and trafficking as well as the role of a number of conserved regions could commence.

Table 5: NS3 mutants that would be investigated during this study.

Protein name	Plasmid	Available	Baculovirus	Available
NS3	pUC-S10	√	Bac-NS3	√
NS3A	pBS-NS3A*	x*	Bac-NS3A	x
NS3-PRO	pFB-NS3-PRO	√	Bac-NS3-PRO	x
NS3-CR	pFB-NS3-CR	√	Bac-NS3-CR	x
NS3-HD1	pFB-NS3-HD1	√	Bac-NS3-HD1	x
NS3-HD2	pFB-NS3-HD2	√	Bac-NS3-HD2	x
NS3-eGFP	pFB-NS3-eGFP	√	Bac-NS3-eGFP	√
NS3A-eGFP	pFB-NS3A-eGFP	√	Bac-NS3A-eGFP	√
eGFP-NS3	pFB-eGFP-NS3	√	Bac-eGFP-NS3	√
NS3ΔC-eGFP	pFB-NS3ΔC-eGFP	√	Bac-NS3ΔC-eGFP	√
N11-eGFP	pFB-N11-eGFP	√	Bac-N11-eGFP	√
NS3-HD1-eGFP	pFB-NS3-HD1-eGFP	√	Bac-NS3-HD1-eGFP	√
NS3-HD2-eGFP	pFB-NS3-HD2-eGFP	√	Bac-NS3-HD2-eGFP	√
eGFP	pFB-eGFP	√	Bac-eGFP	√

*The NS3A plasmid could not be used for generating a baculovirus as described in section 2.3.1.1.

2.3.1 Generation and verification of recombinant baculoviruses

2.3.1.1 Cloning of NS3A into the TOPO® and pDEST vector

In order to prepare the NS3A construct for cloning into the TOPO® vector, NS3A was initially amplified using pBS-NS3A vector as template DNA. A 720 bp fragment was inserted into the TOPO® vector and upon sequencing analysis we found that the cloned NS3A, as well as the pBS-NS3A genes contained an amino acid change from serine to proline at position 602 (results not shown). Since this mutant contains an amino acid with a ring-structure it could affect the secondary protein structure and it was decided to clone NS3A using the pUC-S10 vector as template instead. The pBS-NS3A was, however, still used as a positive control for size comparison during the subsequent cloning steps.

The amplification was performed with NS3A specific primers, NS3ABamF (forward primer) and NS3pEco (reverse primer) which contain *Bam*HI and *Eco*RI restriction sites respectively to facilitate verification of the presence of the insert during subsequent cloning steps. After PCR, amplicons were gel-purified with the Highpure PCR purification kit (Roche) according to the manufacturers' instructions and the full length PCR product was introduced into the TOPO[®] vector with the PCR[®]8/GW/TOPO[®] TA cloning kit according to manufacturers' instructions (Invitrogen). Chemically competent JM109 and DH5α cells were transformed with the TOPO[®] vector and the reactions were plated out on agar plates containing the antibiotic spectinomycin. Approximately 20 colonies were screened for the presence of the vector containing the NS3A insert.

Plasmids were purified with the Roche Highpure PCR purification kit and analysed by electrophoresis in a 1% agarose gel (results not shown). Samples that had a significant size increase relative to the undigested TOPO[®] vector (negative control) were subjected to restriction enzyme digestion for confirmation of the presence of the insert. The TOPO[®] vector contains two *Eco*RI restriction sites flanking the TOPO[®] cloning sites. Plasmids were subjected to restriction enzyme digestion with *Eco*RI and analyzed on a 1% agarose gel. The expected 740 bp gene fragment and a band of approximately 2800 bp in size correlating to the size of the vector fragment were obtained (Figure 6).

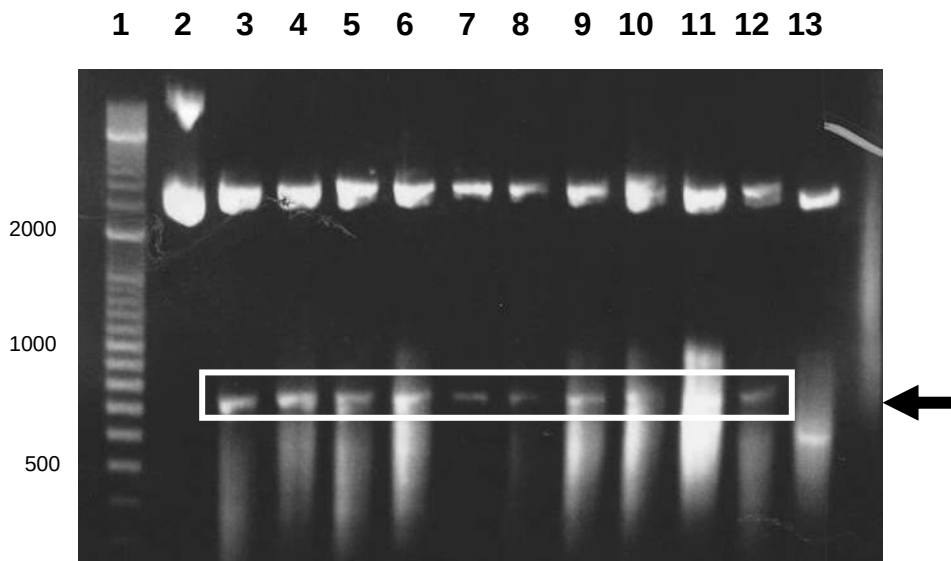


Figure 6. An agarose gel electrophoresis (1% gel) of restriction enzyme digestions (*Eco*RI) used to verify the presence of NS3A in recombinant TOPO[®] vector. Lanes 3-13 each contain a 740 bp fragment corresponding to NS3A and a 2800bp vector fragment. Undigested TOPO[®] vector (lane 2) was included as negative control and O'RangeRuler[™] 100bp+500bp DNA ladder was included as a size marker (lane 1).

Colonies that did not yield the expected size fragments (Figure 6, lane 13) were excluded from further analysis and experimentation.

Before NS3A could be cloned into the pDEST[®] entry vector it was necessary to determine the orientation of the inserts in the TOPO[®] vector. Apart from the previously mentioned *EcoRI* sites, the TOPO[®] vector also contains an *EcoRV* restriction site 127bp downstream of the cloning site. Restriction digestions with *Bam*HI (on the 5' end of the NS3A gene) and *EcoRV* were therefore used to determine the orientation of the insert in the TOPO[®] vector. Recombinants which contain the insert in the correct orientation would then have the *EcoRV* closest to the 3' end of the NS3A gene and upon digestion would yield two DNA fragments: an approximate 850 bp fragment containing the insert and a 2700 bp fragment representing the linearized vector. If the insert occurs in the 3'-5' orientation a band of approximately 127 bp and a vector-gene fragment of 3350 bp would be expected. Four recombinants were tested (Figure 7) and all four were present in the correct orientation.

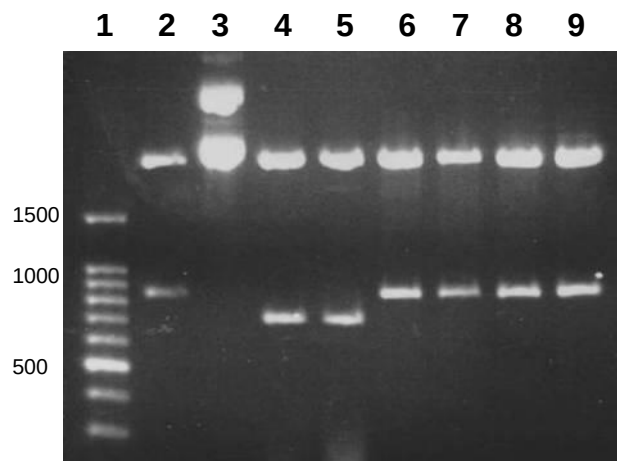


Figure 7. A 2% agarose gel used to determine the orientation of NS3A inserts. The pBS-NS3A vector containing the NS3A fragment (digested with *Bam*HI and *EcoRV*) (lane 2) was included as a positive control, undigested TOPO[®] vector was included as a negative control (lane 3). Lanes 4 and 5 show recombinants which contain the insert (digested with *EcoRI*) and lanes 6-9 show recombinants which contain the insert in the desired 5' to 3' orientation (digested with *Bam*HI and *EcoRV*). O' GeneRuler[™] 100bp ladder (lane 1) was included as size marker.

The four recombinants with the insert in the correct orientation were sequenced to verify the orientation and the presence of a correct reading frame and to screen for the absence of mutations by comparing the sequences to the full-length AHSV-3 gene sequence (results not shown). One clone, designated TOPO-NS3A was selected for subsequent cloning steps.

The cloning of NS3A from the TOPO[®] entry vector into the pDEST[™]8 destination vector relies on the site-specific recombination between these two vectors. TOPO-NS3A was used to set up an LR recombination reaction (as described in section 2.2.16). Chemically competent DH5 α cells were transformed with the LR reaction mix and plated out on agar plates containing the antibiotic ampicillin. Approximately 20 colonies were screened for the presence of the pDEST[™]8 vector containing the NS3A insert by means RE digestion of purified plasmid with *Bam*HI and *EcoRI*.

Samples were analysed on 1% agarose gels (Figure 8). All recombinants revealed a band of 720 bp which corresponds to the size of the NS3A insert as well as a 6500 bp band which corresponds to the size of the linearized pDEST™8 vector.

One of the recombinants designated pDEST-NS3A, could now be used to transform DH10Bac™ *E. coli* cells to generate recombinant bacmids for expression in Sf9 insect cells.

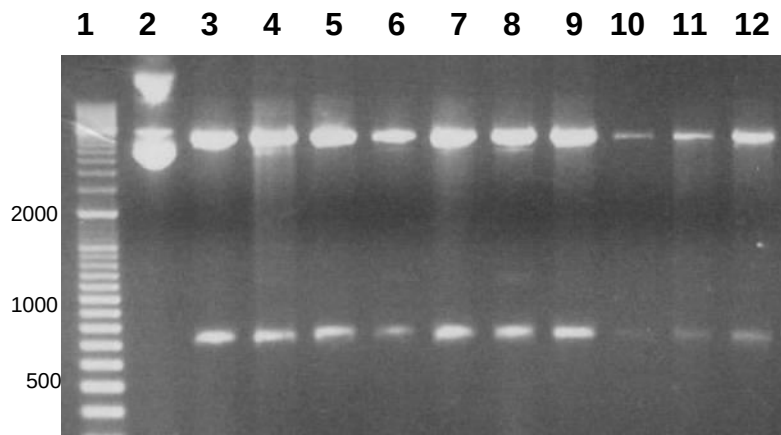


Figure 8. A 1% agarose gel used to confirm the presence of the NS3A insert in the pDEST™8 destination vector. Lanes 3-12 show samples after RE digestion with *Bam*HI and *Eco*RI. Undigested pDEST™8 vector was included as negative control (lane 2) and O'RangeRuler™ 100bp+500bp DNA ladder was included as size marker (lane 1).

2.3.1.2 Transformation of DH10Bac™ cells and transfection of Sf9 insect cells with bacmid DNA

DH10Bac™ cells contain a baculovirus vector (bacmid) with a mini-*att*Tn7 target site as well as a helper plasmid. pDEST™8 vectors contain a mini-Tn7 element. During transformation, a transposition reaction occurs between the pDEST™8 vector mini-Tn7 element and the baculovirus vector mini-Tn7 target site in the presence of transposition proteins supplied by the helper plasmid (Invitrogen Life Technologies). The gene of interest is then transposed into the baculovirus vector to produce a recombinant bacmid.

Electro- or chemically competent DH10Bac™ cells were transformed with pDEST-NS3A, pFB-NS3-PRO, pFB-NS3-CR, pFB-NS3-HD1 and pFB-NS3-HD2. Transformed cells were plated on agar plates containing the antibiotics gentamycin, kanamycin and tetracyclin as well as IPTG and Xgal. After incubation white colonies were selected and restreaked on similar plates to confirm the white phenotype. Approximately 20 colonies per transformation were selected, from which bacmid genomic DNA was isolated and analysed on a 1% agarose gel for the presence of high molecular weight DNA (results not shown).

For each of the five abovementioned constructs, purified bacmid DNA was used for transfecting Sf9 insect cells. The insect cells were incubated for 48 hours at 27°C. Viral supernatants were plaque purified and the supernatant from single plaques were used to infect Sf9 cells at a low multiplicity of infection (MOI) to amplify the virus. After four rounds of amplification, viral titres of the baculovirus stocks were determined. The remaining nine constructs for which baculovirus stocks were available, were re-amplified by infecting Sf9 cells at a MOI of 0.1. After amplification the viral titres were determined (Table 6).

Table 6: Viral titres from recombinant baculoviruses expressing the various NS3 proteins in Sf9 cells.

Stock	Viral titre (pfu/ml)
Bac-NS3	3.3×10^7
Bac-NS3A	1.9×10^7
Bac-NS3-PRO	5.2×10^7
Bac-NS3-CR	1.4×10^8
Bac-NS3-HD1	6.3×10^7
Bac-NS3-HD2	1.4×10^8
Bac-NS3-eGFP	8.5×10^6
Bac-NS3A-eGFP	3.1×10^7
Bac-eGFP-NS3	4.2×10^6
Bac-NS3 Δ C-eGFP	2.7×10^7
Bac-N11-eGFP	3.1×10^7
Bac-NS3-HD1-eGFP	2.7×10^7
Bac-NS3-HD2-eGFP	3.3×10^7
Bac-eGFP	5.7×10^7

In order to confirm the expression of the NS3 proteins in Sf9 cells, cells were harvested and subjected to SDS-PAGE. NS3 is normally expressed at low levels in host cells and therefore Western blot analysis with anti-NS3 antibodies was necessary to detect and quantify the protein expression levels (Figure 9).

Cell lysates were analysed using SDS-PAGE (in a 15% gel. Western blots were performed with anti-eGFP antibodies (diluted 1/4000) or anti-NS3 antibodies (diluted 1/100) for the eGFP- or non-eGFP tagged constructs respectively, to confirm the expression of the NS3 proteins (Figure 9).

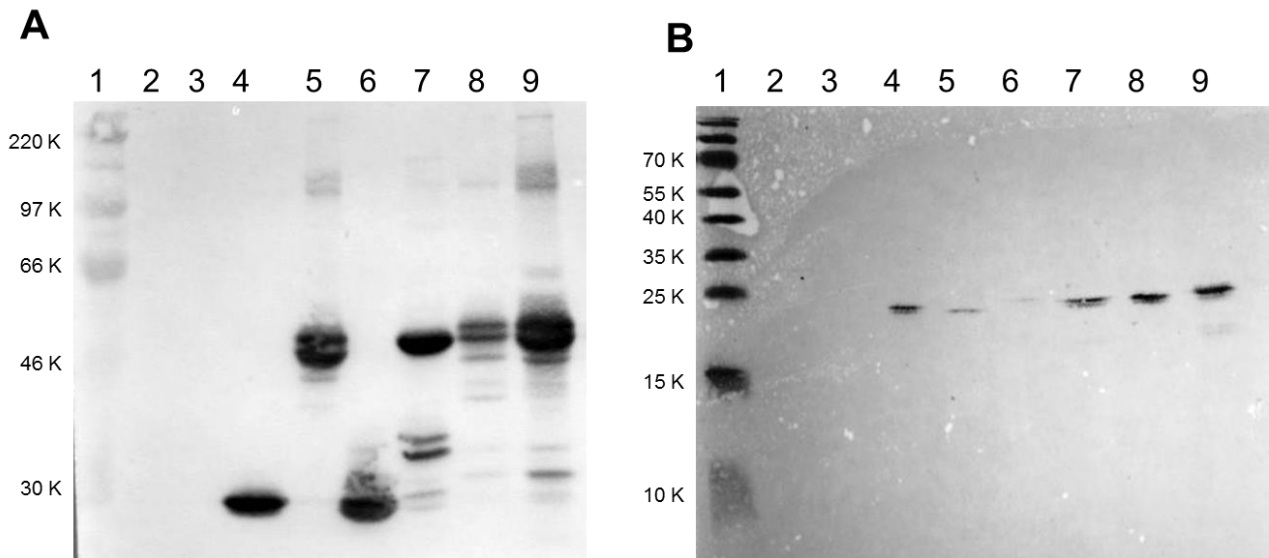


Figure 9. Western blots with anti-eGFP (A) and anti-NS3 serum (B). Blot A: Cells were mock infected (lane 2), or infected with wild-type baculovirus (lane 3), Bac-eGFP (lane 4), Bac-NS3 Δ C-eGFP (lane 5), Bac-N11-eGFP (lane 6), Bac-eGFP-NS3 (lane 7), Bac-NS3A-eGFP (lane 8) and Bac-NS3-eGFP (lane 9) whilst RPN 756 size marker was included in lane 1. Blot B: Cells were mock infected (lane 2), infected with wild-type baculovirus (lane 3), Bac-NS3 (lane 4), Bac-NS3A (lane 5), Bac-NS3-HD1 (lane 6), Bac-NS3-HD2 (lane 7), Bac-NS3-PRO (lane 8), Bac-NS3-CR (lane 9) and PAGE RULER™ was included as size marker (lane 1).

From the Western blots it was evident that the eGFP-tagged proteins were expressed at much higher levels than the non-eGFP proteins. For NS3 Δ C-eGFP, NS3-eGFP and NS3A-eGFP the expected 51kDa band as well as an additional protein band of approximately 50kDa was observed. These findings correlate with that of Hatherell (2007) upon initial generation and analysis of these constructs. It was found that these additional bands were not due to glycosylation however the reason for these bands remains unclear. The second band was not present for the eGFP-NS3 protein. The expected 27kDa bands were observed for the WT eGFP and N11-eGFP proteins. The NS3-HD1-eGFP protein and the NS3-HD2-eGFP could not be detected on a Western blot.

All of the non-eGFP proteins showed expected size bands of approximately 24 kDa. NS3-HD1 protein was present in notably lower levels than proteins NS3, NS3A, NS3-PRO, NS3-CR and NS3-HD2.

With the exception of the two eGFP-tagged hydrophobic domain mutants, it was therefore established that all the constructs were successfully expressed in Sf9 cells. Since both of the eGFP tagged hydrophobic domain mutants had reasonably high viral titres it was decided that they would still be included in subsequent experiments. We could now start the visualisation studies to establish the localisation of the different NS3 proteins constructs and determine the effects of mutations on different conserved regions or residues by means of confocal microscopy.

2.3.2 Establishing a standard protocol for confocal microscopy

2.3.2.1 Optimisation of different fixatives for GFP-labelled vs non-GFP NS3 constructs

There are various protocols available in literature that are used for fixing cells prior to immunolabelling of proteins for visualisation in confocal microscopy, which have to be tested and optimised for each individual application. We compared two of the most commonly used protocols, i.e. fixing with methanol/acetone versus fixing with a 4% paraformaldehyde (PFA) solution.

For analysis of eGFP fusion proteins, Sf9 cells were grown on glass coverslips and were infected with Bac-NS3-eGFP. At 24 hpi cells were fixed using either methanol/acetone or 4% PFA. Unfixed cells were washed once in 1 x PBS buffer. Coverslips were mounted on microscope slides for visualisation by the confocal microscope (Figure 10).

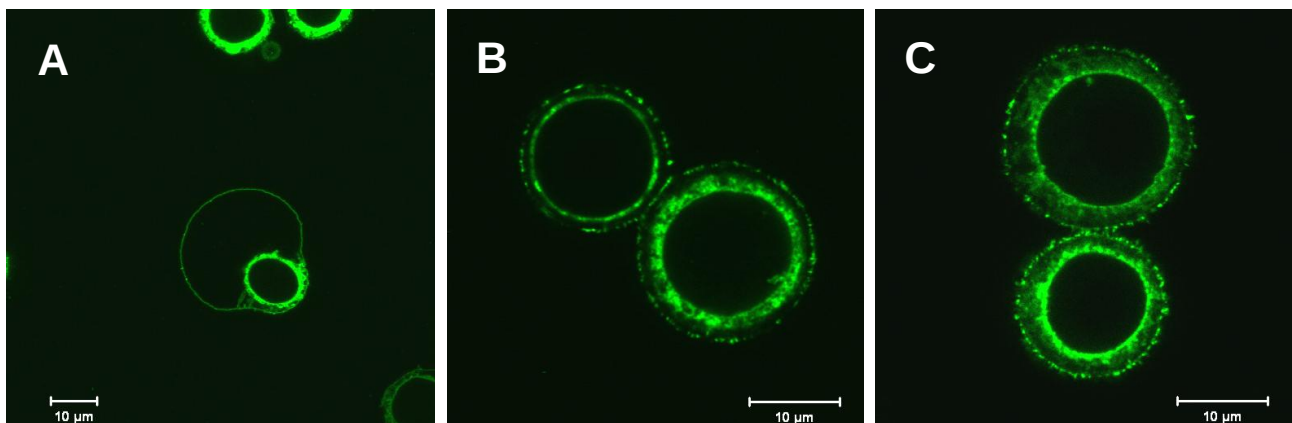


Figure 10. Confocal images of Sf9 cells infected with Bac-NS3-eGFP. Sf9 cells were unfixed (A) or fixed with 1:1 methanol/acetone solution (B) or fixed with a 4% formaldehyde solution (C). All scale bars represent 10 µm.

Upon comparing the results of the unfixed cells with the two different fixation methods it was observed that unfixed cells started to swell and cell membranes ruptured, probably due to the hypotonic environment during the washing step. Because of this, it was decided that cells expressing eGFP-fusion proteins would also need to be fixed before analysis. Both fixation methods yielded similar quality results with no influence on the fluorescence of NS3-eGFP.

The same protocols were compared for non-eGFP tagged proteins. Cells were mock infected, or infected with wild-type baculovirus or Bac-NS3. Cells were either fixed with methanol/acetone or treated with 4% PFA and Triton X-100. This permeabilises the cell membrane prior to immunolabelling. Proteins were then immunolabeled with anti-NS3 (primary antibodies) and Alexa Fluor488™ (secondary antibodies) (Figure 11).

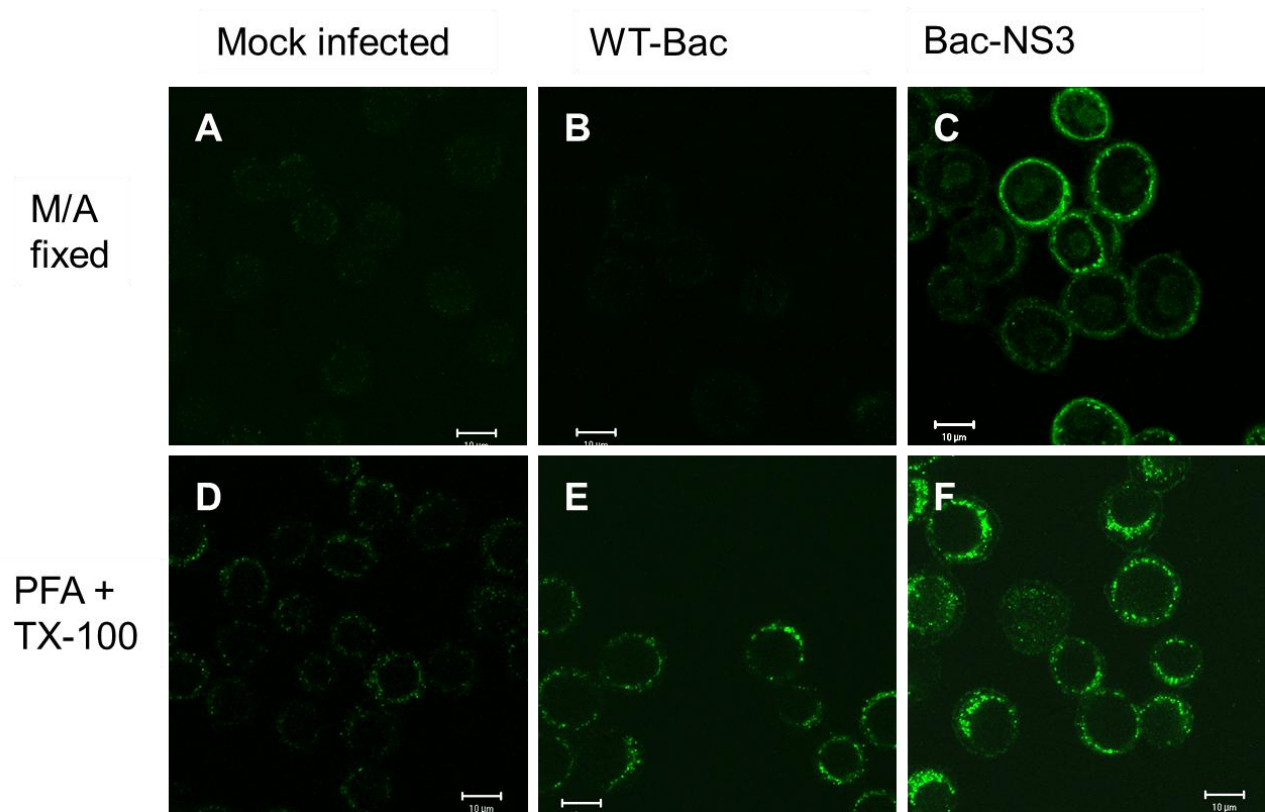


Figure 11. Confocal images of immunolabelled Sf9 cells. Mock (A and D), wild-type baculovirus infected (B and E) and Bac-NS3 infected (C and F) cells were either fixed with 1:1 methanol/acetone (A, B and C) or 4% PFA (D, E and F) before immunolabelling with anti-NS3 (primary antibody) and Alexafluor 488™ (green secondary antibody). All scale bars represent 10 µm.

Neither of the fixatives influenced the immunolabelling and/or the fluorescence levels of the proteins and no quenching was observed. However, much more background labelling was observed for the controls after fixing with 4% PFA and permeabilisation with Triton X-100 (Figure 11 D and E). This could be due to the auto-fluorescent nature of detergents such as Triton X-100. The NS3 expressing cells that were fixed with 4% PFA and Triton X-100 had a slightly more grainy appearance when compared to cells that were fixed with methanol/acetone (Figure 11 C and F). The grainy appearance could be explained by the cross-linking of proteins caused by the 4% PFA. Since 1:1 methanol/acetone takes less time to prepare and the fixation occurs more rapidly we decided that all other experimentation would be performed using this method. Once the fixation method was optimised we could go on to determine the time post infection at which optimum expression of the constructs occur for use in confocal analysis.

Since expression under control of a polyhedrin promoter (as in the case of our recombinant baculovirus proteins) only starts from approximately eighteen hours post infection and cells in cultures expressing NS3 start dying from about 27 hours after infection with no viable cells visible at 48 hpi, we decided to conduct a time trial in order to establish what time post infection would yield good protein expression and a large proportion of viable cells. One of the characteristic features of Sf9 insect cells is the prominent large nucleus. In order to visualise the localisation of

the various NS3 proteins relative to the cell nucleus, 4'6'-diamidino-2-phenylindole (DAPI) was added to the cells before visualisation. DAPI inserts into the minor groove of DNA by the displacement of water molecules. Due the high fluorescent signal of the DAPI the whole nucleus appears blue.

For the time trial, Sf9 cells were infected with Bac-NS3-eGFP at an MOI of 10 and were analysed at 18, 24, 27, 30, 36, 42 and 48 hpi without (not shown) or with (Figure 12) the presence of DAPI. An MOI of 10 was used for infection to ensure a synchronized infection. Cells were fixed with 1:1 methanol/acetone at the different times post infection and analysed by confocal microscopy. The time trial was also repeated for cells fixed with 4% PFA (results not shown) for verification, and similar results were obtained.

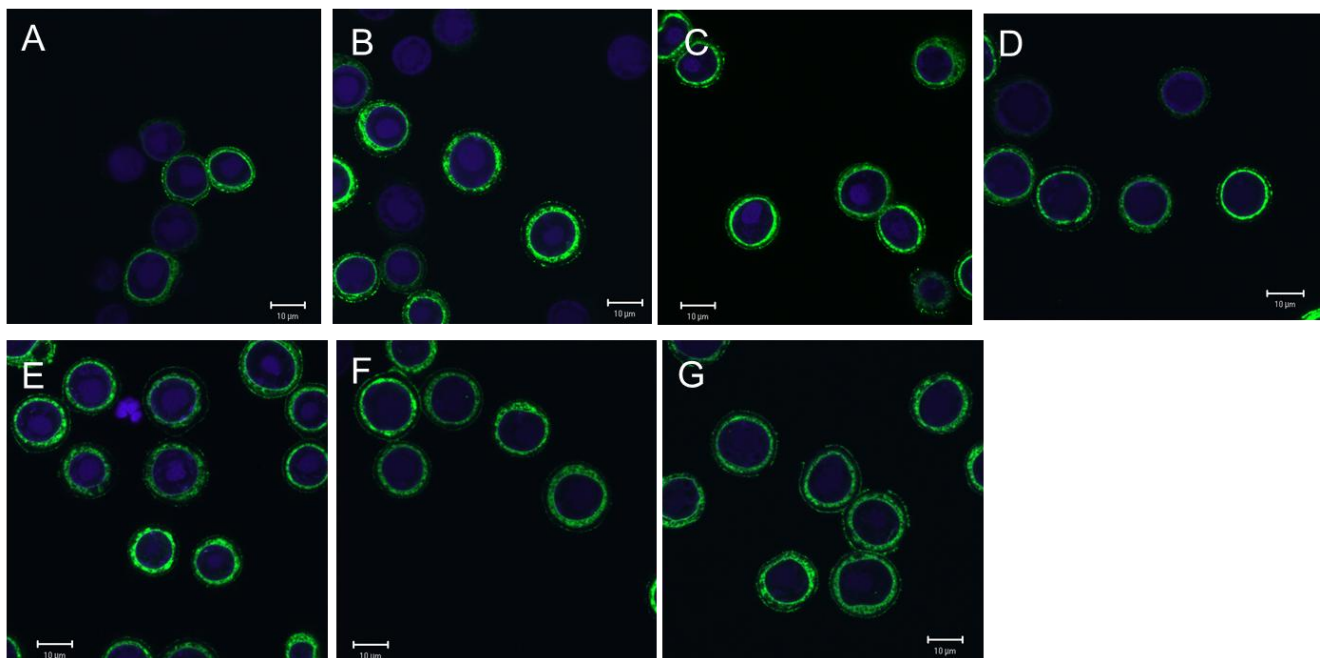


Figure 12. Confocal microscopy images of Sf9 cells infected with Bac-NS3-eGFP also labelled with a nuclear dye, DAPI at 18 (A), 24 (B), 27 (C), 30, (D), 36 (E), 42 (F) and 48 (G) hpi. Scale bars represent 10 μm.

All infected cells revealed that NS3-eGFP showed the same expression pattern, regardless of the time after infection. At 18 hours post infection the expression levels of NS3-eGFP seemed to be lower than at the later times of analysis. As previously mentioned this is probably due to the late onset of expression under control of the baculovirus polyhedrin promoter. Between 24, 27, 30 and 36 hpi no differences in expression levels could be observed when the whole field of cells were taken into account. At 42 hpi there were a few cells that showed no difference to the earlier times however across the field there was an overall decrease in the amount of fluorescence in the infected cells. Whilst few cells again showed no difference to earlier assay times, at 48 hpi the number of viable cells across the field were considerably less. It was therefore decided that all further experiments would be carried out at 27 hpi. A previous study noted that GFP fusion proteins

decrease the cytotoxic effect that NS3 normally exerts on cells and that the number of viable cells at 48 hpi decreased by 50% (Hatherell, 2007). In the presence of DAPI, photobleaching of the green fluorescent protein was observed after multiple scans of the same cell. This was observed for both methanol/acetone and PFA fixed cells. Similar results were found for cells infected with other GFP-tagged proteins in the presence of DAPI. The reason for the photobleaching is not entirely clear, but one possible reason could be due to the excitation wavelengths of the green and the blue light within close range of each other. When the blue light emission scans over the cell it results in fading of the more light sensitive green fluorescent protein.

When later in this study an additional cellular marker within the red light range (Alexafluor 594™ or 647™) was added for labelling the Golgi apparatus or endoplasmic reticulum, no photobleaching of the eGFP-tagged proteins was observed (see results 2.3.14.2). Once all the conditions and methods for preparing samples had been optimised we could proceed to compare the effects of different amino acid substitutions or deletions in conserved regions of NS3 on protein localisation.

The LSM510 Meta Confocal Microscope has a number of unique functions which proved very useful in studying the different localisation patterns of each of the recombinant NS3 proteins. These functions will be explained in terms of the NS3-eGFP protein

2.3.2.2 Applications of the LSM 510 Meta Confocal Microscope used for comparison of the various NS3 proteins

2.3.2.2.1 Z-stacking

Normal light microscopes allow the visualisation of cells or samples only in a single plane. Although this feature can still provide useful information, the data obtained from a single focal plane only represents what is happening in a specific part of the cell at a given time. The confocal microscope has an advanced function that allows the user to define the outer margins of a cell and then scan through the sample for a set number of intervals to obtain a number of slices of a single cell (Figure 13).

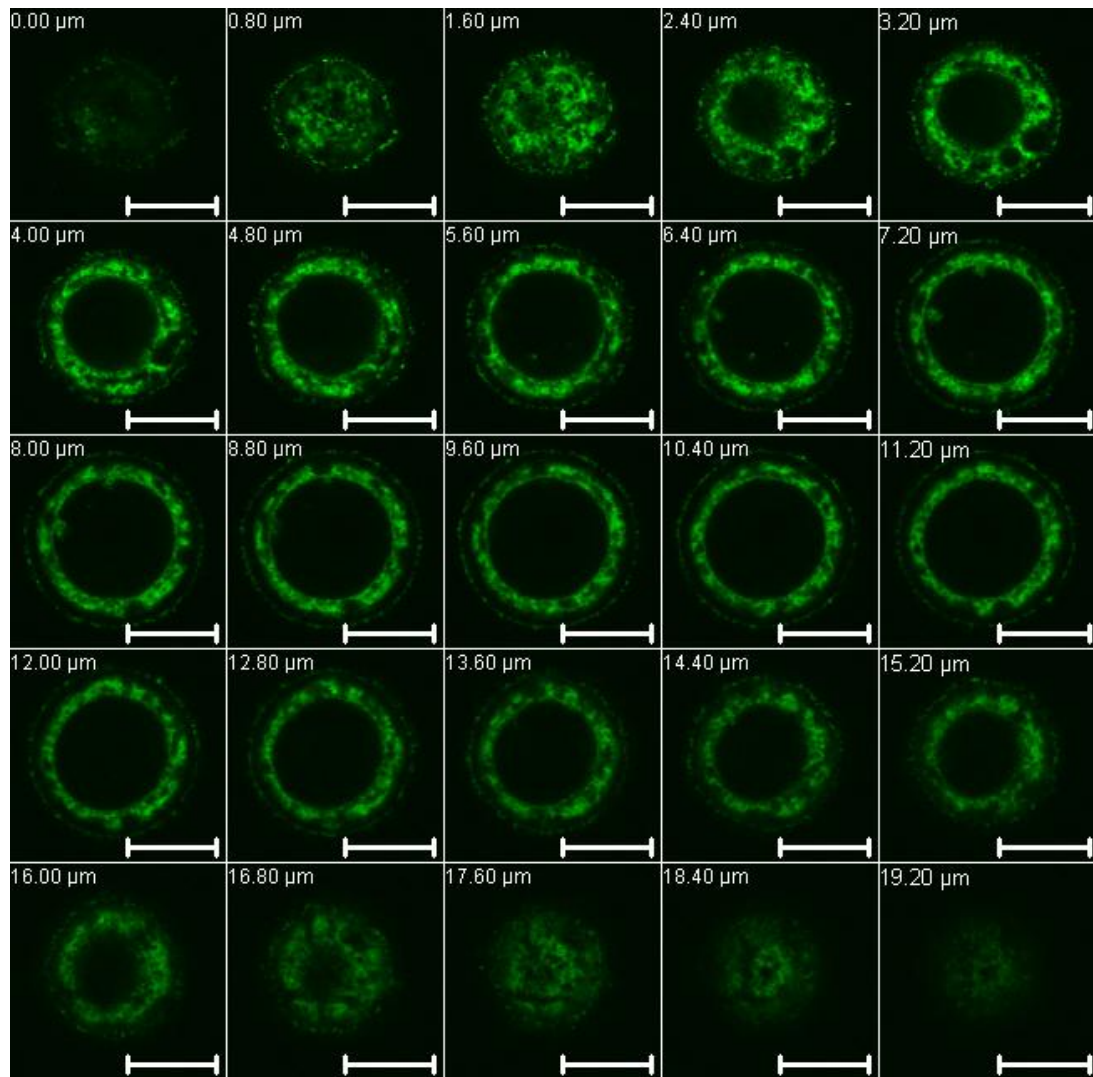


Figure 13. A Z-stack gallery showing a series of slices at predetermined intervals through a single Bac-NS3-eGFP infected Sf9 cell. Scale bars represent 10 μm .

Using the Z-stack information, the LSM software can reconstruct a three-dimensional image of the scanned cell (Figure 14).

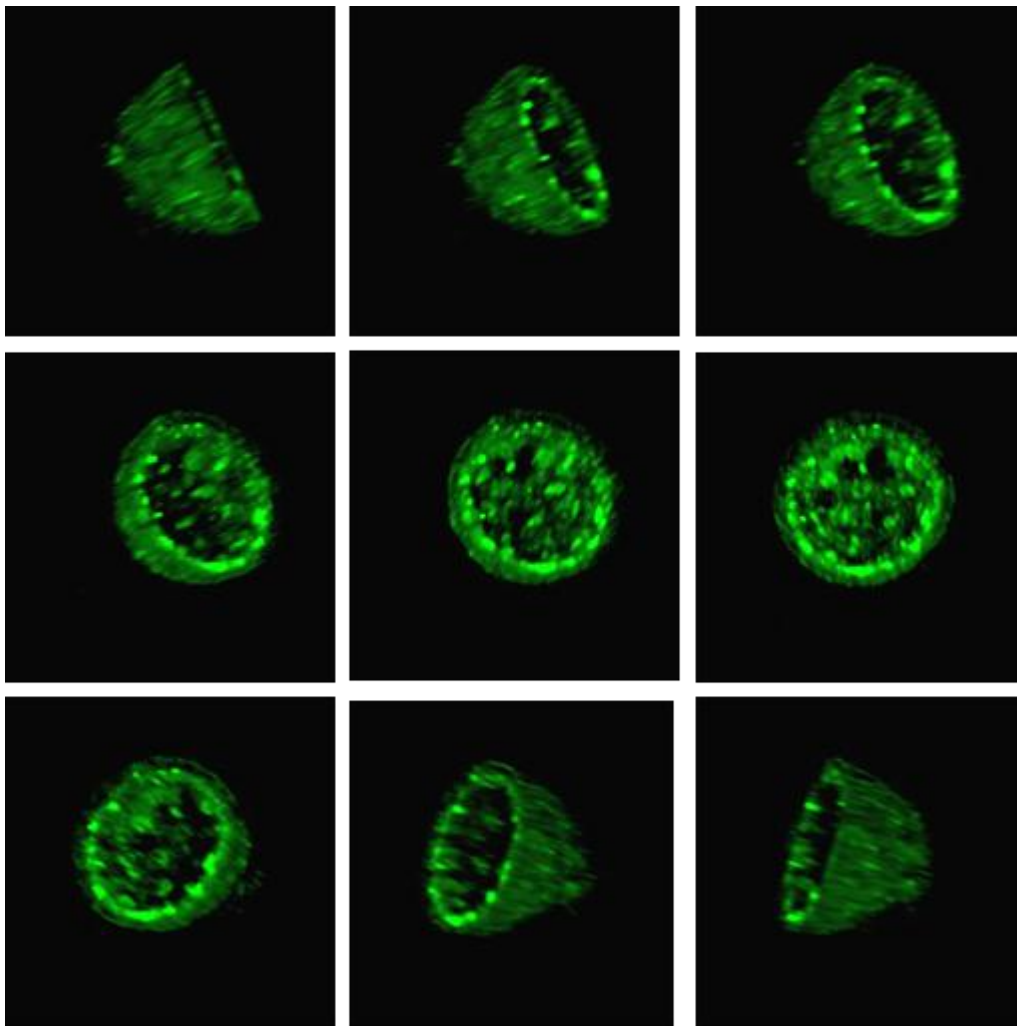


Figure 14. Three dimensional reconstruction of a Bac-NS3-eGFP infected Sf9 cell (cut in half) as it is rotating around its Y-axis. The 3-D imaging relies on the data obtained from a Z-stack.

With the 3-dimensional images of the infected Sf9 cells it was possible to determine the exact expression profile of the various mutant proteins. This function allows the user to manipulate the rotation of the 3-D cells in various directions so that the expression profile of the proteins is visible from any possible angle (see Appendix A for supplementary video data). Z-stacking was mainly applied to establish the localisation of the NS3 mutant proteins to the cell membrane. It was also used to determine whether the intranuclear bodies which are present in NS3A and the NS3-CR expressing cells are attached to the nuclear membrane or not membrane associated (see Results section 2.3.3.1).

Unfortunately Z-stacking and consequent 3-D imaging was not possible for all the samples, since continuous scanning of the same cell occasionally resulted in photobleaching depending on the mutant protein under study. This problem was mainly limited to cells that also contained DAPI, as well as the cells that were expressing mutant proteins that emitted very low fluorescence signals (NS3-HD1 and NS3-HD2).

With access to all these LSM tools, we could now go on to compare whether mutations in the NS3 protein altered its localisation in the presence or absence of an eGFP tag.

2.3.3 Comparison of different NS3 protein expression profiles in Sf9 insect cells

2.3.3.1 Comparing the intracellular distribution of 14 NS3 constructs in Sf9 insect cells

Fourteen different recombinant baculoviruses expressing different forms of the AHSV-3 NS3 protein were available (Table 5). By comparing these different constructs, we aimed to answer two main questions. Firstly, we wanted to determine what the effect of the eGFP-tag would be on the localisation and trafficking of the NS3 proteins. Secondly we wanted to determine the effects of mutations in the gene encoding NS3 resulting in amino acid substitutions and deletions in the different domains of NS3, on protein localisation and trafficking. In order to answer these questions we grouped the constructs together according to their mutations for analysing and comparing the results.

For each experiment that was performed, uninfected (mock infected) Sf9 cells as well as Sf9 cells infected with wild-type baculovirus (WT-Bac) were included as negative controls. Wild-type eGFP (WT-eGFP) was also included in all the experiments as a positive control. A cell infected with WT-eGFP would typically present with a uniform distribution of eGFP throughout the whole cell. The mock infected and wild-type baculovirus infected Sf9 cells shown in Figure 15 A and B is representative of the results that were obtained for the negative controls after immunolabelling with anti-NS3 serum.

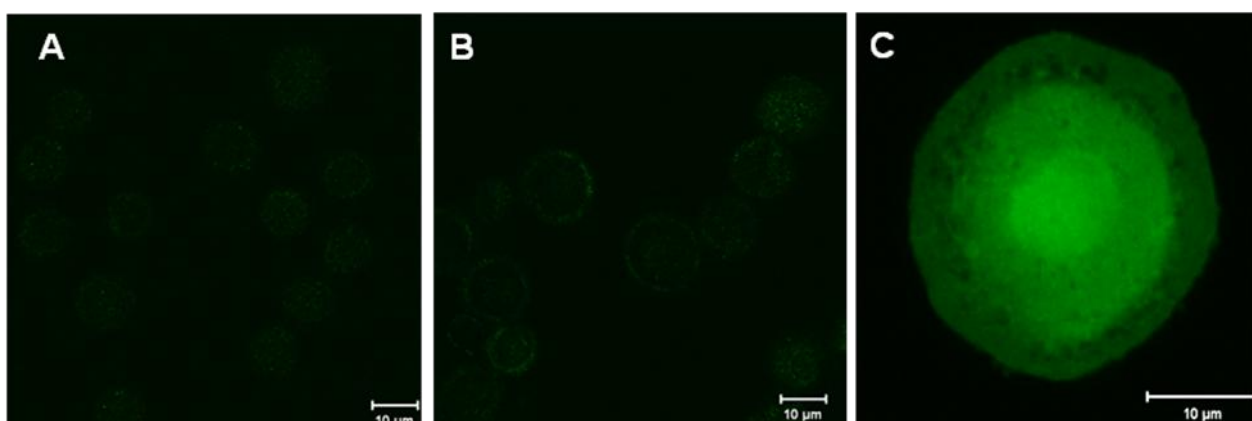


Figure 15. Mock infected (A) cells and Sf9 cells infected with WT-Bac (B) were included as negative controls for confocal microscopy whilst cells expressing WT-eGFP protein served as positive control (C). Scale bars represent 10 µm.

If the fluorescence signal for the negative controls was higher than that shown in Figure 15, then all results from the experiment were disregarded and the experiment was repeated. For the comparative study of the NS3 proteins, all experiments consisted of at least two technical repeats

and between four and six biological repeats to obtain a valid representation of the range (if any) of results for each protein.

Our first aim was to determine whether the presence of an eGFP tag would influence protein folding and subsequently, protein localisation and trafficking. To address this question we compared immunolabelled wild-type NS3 protein to constructs which contain an eGFP-tag on the C- or N-terminus, designated NS3-eGFP and eGFP-NS3 respectively.

Sf9 cells were infected with recombinant baculoviruses, Bac-NS3, Bac-NS3-eGFP and Bac-eGFP-NS3 respectively. Cells that expressed the eGFP-tagged proteins were fixed and mounted for visualisation. The cells expressing the wild-type NS3 protein were fixed and incubated with anti-NS3 primary antibodies. Alexa Fluor488™ (green fluorescence) was used as a secondary antibody (Figure 16).

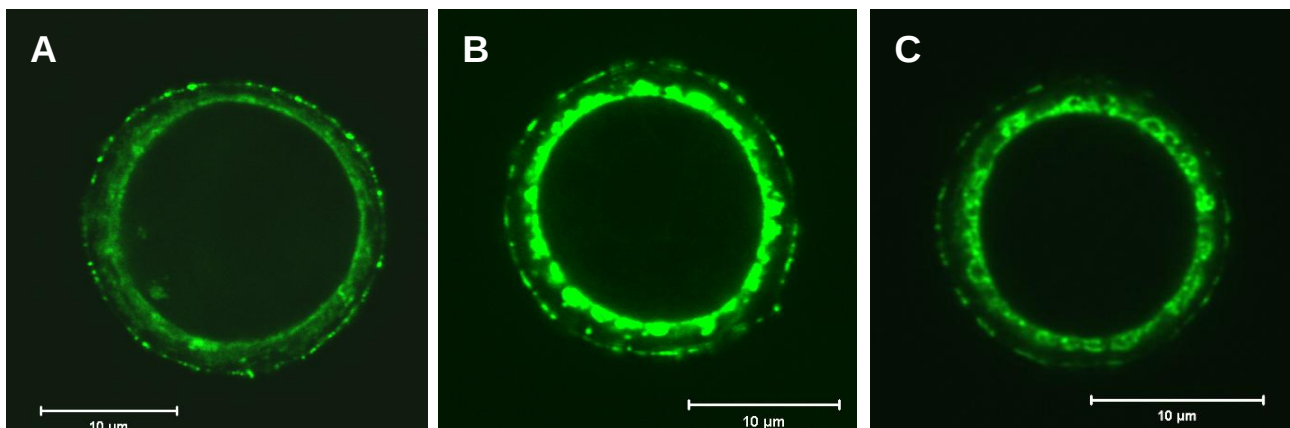


Figure 16. Confocal images (100x) of Sf9 cells infected with Bac-NS3 (immunolabelled) (A) compared with Bac-NS3-eGFP (B) and Bac-eGFP-NS3 (C) at 27 hpi. Scale bars represent 10 µm.

The immunolabelled NS3, NS3-eGFP and eGFP-NS3 all showed a similar punctate perinuclear, cytoplasmic localisation as well as localisation to the nuclear membrane and an uneven distribution in the outer plasma membrane. The strong, irregular cell membrane signal of the NS3-eGFP and eGFP-NS3 was clearly visible in the 3-D reconstruction of the cell Z-stack data whilst the perinuclear localisation gives the cell a hollow appearance (Appendix A - supplementary video). The perinuclear signal could be indicative of localisation to the ER and Golgi apparatus. On rare occasions, single Bac-NS3 infected Sf9 cells also showed intranuclear bodies. The reason for these intranuclear bodies remains unclear. The eGFP fusion proteins emitted significantly higher fluorescence signals than the immunolabelled NS3. The localisation and trafficking of the full-length NS3 protein was not influenced by the eGFP-tag on either the N- or C-terminal of the protein. Based on these findings, the eGFP tag could provide a useful non-invasive tool for studying the localisation and trafficking of NS3 proteins.

2.3.3.2 Comparing the localisation of deletion mutant proteins

When a part of a protein is highly conserved it normally suggests a functional role for those amino acids. Since NS3 is believed to be involved in trafficking we wanted to investigate the roles of the highly conserved regions in NS3 previously identified (Van Staden, Huismans, 1991) by mutation analysis. In wild-type AHSV infected cells the segment 10 mRNA produces the full-length NS3 protein as well as a truncated protein, NS3A, which is encoded from a second in-frame starting codon. In BTV it is proposed that the first thirteen amino acids on the N-terminus of the NS3 protein interact with cellular proteins to enable trafficking to the cell membrane whilst the C-terminal amino acids interact with newly assembled virus particles to be transported for viral release. We therefore wanted to see whether NS3A (which lacks the first 11 amino acids of the full-length protein) and the NS3 Δ C-eGFP (which lacks the last 14 amino acids of the full-length protein including the dileucine motif), would localise differently to wild-type NS3 in Sf9 cells. The N11-eGFP protein, which contains only the first eleven amino acids of NS3 fused to eGFP was also included, to determine whether these eleven amino acids are responsible for trafficking to the plasma membrane. Bac-NS3A, Bac-NS3A-eGFP, Bac-NS3 Δ C-eGFP and Bac-N11-eGFP infected Sf9 cells were fixed at 27 hpi, immunolabelled in the case of NS3A, and mounted for analysis by confocal microscopy (Figure 17).

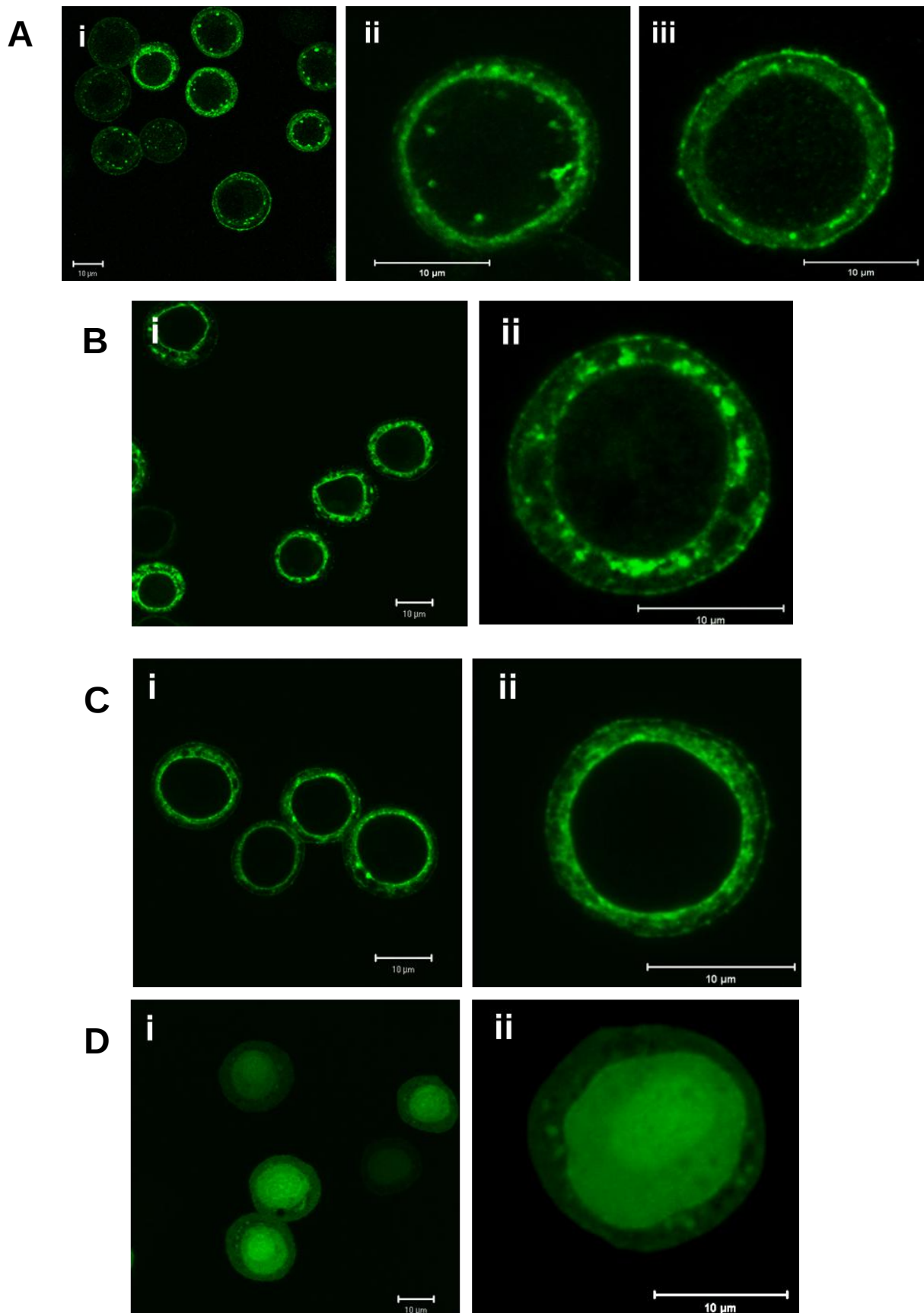


Figure 17. Confocal images (100x) of Sf9 insect cells expressing NS3A (immunolabelled) (A) compared with NS3A-eGFP (B), NS3ΔC-eGFP (C), N11-eGFP (D). In each panel on the left a wide field image is shown. Each panel on the right shows a single cell. Scale bars represent 10 μm.

The untagged NS3A protein showed two different localisation patterns in infected cells. In approximately 80% of the Bac-NS3A infected cells it appeared that little of the protein was trafficked to the plasma membrane (Figure 17 Ai) and intranuclear bodies (INB) were present, however the bulk of the proteins still appeared to be punctately localised in the perinuclear cytoplasm. The intranuclear bodies that were observed in the Bac-NS3A infected cells appeared to project into the cell nucleus and from the data of both the single plane images and the 3-D images and video data it was unclear whether these INBs were membrane associated. In 20% of the Bac-NS3A infected cells the localisation of the NS3A proteins looked similar to the wild-type NS3 with the punctate perinuclear and plasma membrane distribution. NS3A-eGFP proteins showed a similar protein distribution to NS3 in the cytoplasm however only occasionally did NS3A-eGFP localise unevenly in the plasma membrane. Again the NS3A-eGFP-tagged proteins had higher fluorescence signals compared to the untagged immunolabelled NS3A proteins. In contrast to NS3, the presence of the eGFP tag does seem to influence the localisation and trafficking of NS3A.

NS3 Δ C-eGFP mutant proteins all showed a similar punctate perinuclear (which suggests ER and Golgi associated localisation) and plasma membrane distribution, as the wild-type NS3 protein. The N11GFP protein showed a uniform, non-localised distribution throughout the cell, similar to that of the wild-type eGFP protein (Figure 15). This could be due to the eleven amino acid peptide being too short to counter the soluble effects of the eGFP tag. Alternatively, it is possible that the trafficking of NS3 is not influenced by the first eleven amino acids but mainly depends on other conserved motifs. The video data obtained for the above mentioned constructs correlate with the 2-D confocal images.

2.3.3.3 Comparing the localisation of substitution mutants

In order to visualise the localisation of NS3-PRO, NS3-CR, NS3-HD1, NS3-HD2, NS3-HD1-eGFP and NS3-HD2-eGFP, Sf9 cells were infected at an MOI of 10. At 27 hpi cells were fixed and mounted for NS3-HD1-eGFP and NS3-HD2-eGFP and immunolabelled in the case of NS3-PRO, NS3-CR, NS3-HD1 and NS3-HD2 and mounted for visualisation under the confocal microscope. Before considering the results it is noteworthy to mention that the eGFP-tagged hydrophobic domain mutants, NS3-HD1-eGFP and NS3-HD2-eGFP presented with very low levels of fluorescence (Figure 18).

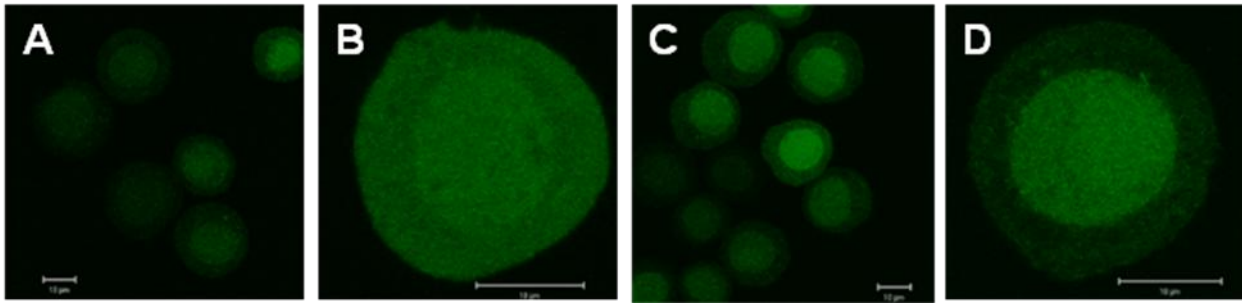


Figure 18. Confocal images showing the low fluorescence signal detected for eGFP-tagged hydrophobic domain mutants. A and B show NS3-HD1-eGFP whilst C and D show NS3-HD2-eGFP. Scale bars represent 10 μ m.

For both the NS3-HD1 and NS3-HD1-eGFP mutants we found that these proteins showed very low levels of fluorescence (Figure 19) which can be attributed to low expression levels that were observed with Western blots. The NS3-HD1 mutant presented variable results with every repetition of the experiment. Overall we observed a punctate cytoplasmic localisation different to the WT NS3. The NS3-HD1 proteins were never observed to localise to the nuclear membrane and in the cases where NS3-HD1 was observed in the plasma membrane, it appeared to have a smooth distribution regularly throughout the membrane. In the case of HD1-eGFP a uniform homogenous distribution throughout the cytoplasm and cell nucleus, similar to that of wild-type eGFP, was observed.

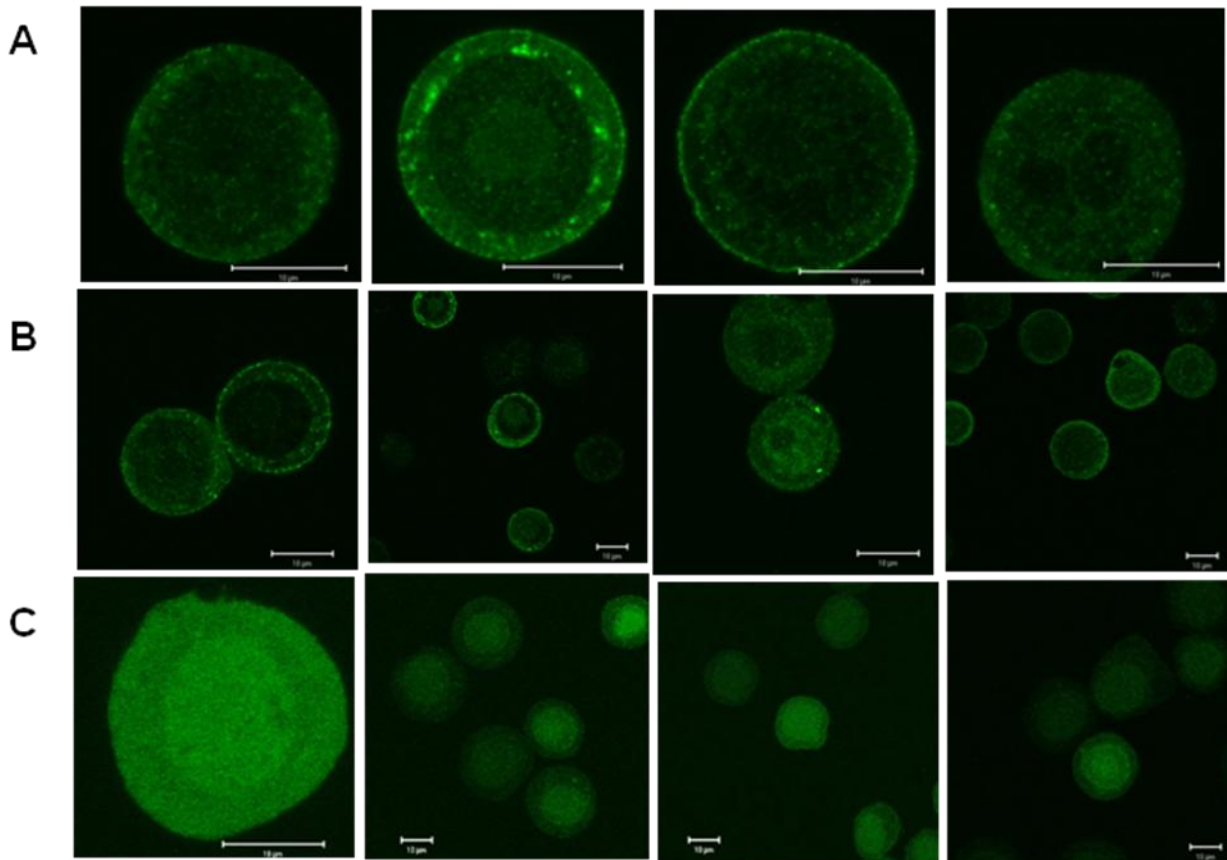


Figure 19. Confocal images (100x) of NS3 proteins that carry mutations in the first hydrophobic domain. NS3-HD1 mutants (A and B) were compared with NS3-HD1-eGFP (C) at 27 hpi. Scale bars represent 10 μ m. For comparative purposes, the brightness and contrast settings for these images were adjusted.

From the confocal analysis, NS3-HD2 appeared to have a more homogenous distribution throughout the cell cytoplasm (Figure 20). On some occasions a homogenous distribution inside the nucleus was also observed. In rare instances when NS3-HD2 was observed to localise to the plasma membrane, it had an irregular distribution, similar to the WT NS3 protein. Like the NS3-HD1-eGFP, the NS3-HD2-eGFP proteins also showed a uniform distribution throughout the whole cell similar to the wild-type eGFP protein.

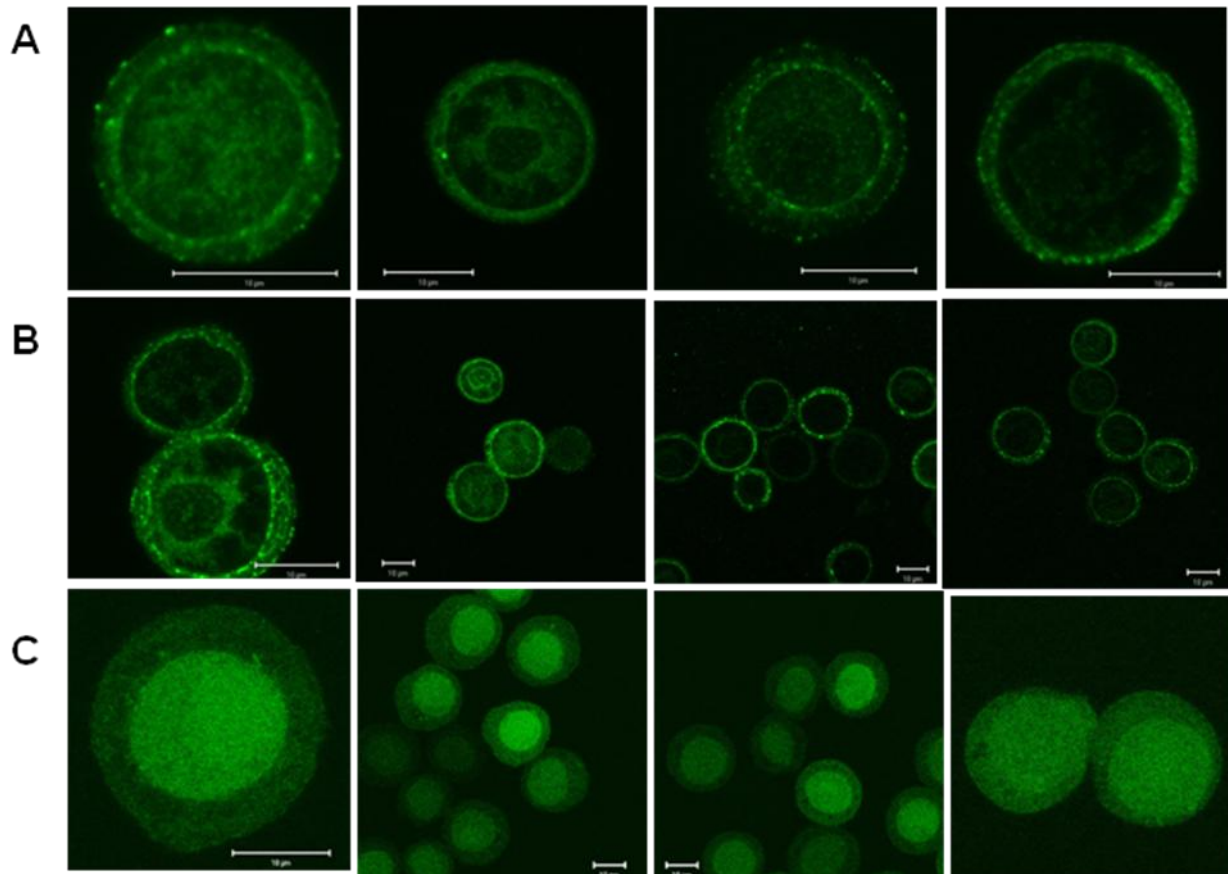


Figure 20. Confocal images (100x) of NS3 mutants encoded by genes that contain mutations in the region encoding the second hydrophobic domain. NS3-HD2 mutants (A and B) were compared with NS3-HD2-eGFP (C) at 27 hpi. Scale bars represent 10 µm.

Western blot analysis of cell lysates following infection with NS3-HD1-eGFP and NS3-HD2-eGFP was performed with anti-eGFP antibodies (Figure 21). The NS3-HD1-eGFP and NS3-HD2-eGFP were both observed as a single band of approximately 27 kDa, approximately the size of the WT eGFP protein. These results confirm cleavage of both NS3-HD1-eGFP and NS3-HD2-eGFP proteins into an NS3 protein and an eGFP protein. For both the NS3-HD1-eGFP and NS3-HD2-eGFP mutants the fluorescence signals were too low for reconstructing 3-D videos.

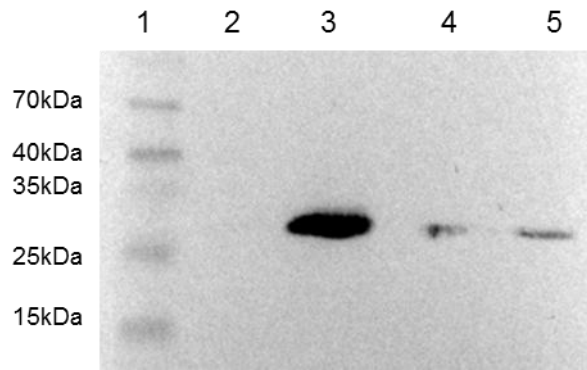


Figure 21. Anti-eGFP (1/100) western blot showing WT eGFP (lane 3) and cleavage products of NS3-HD1-eGFP (lane 4) and NS3-HD2-eGFP (lane 5). PAGE RULER™ was included as size marker (lane 1) and mock infected cells (lane 2) were included as negative control.

These results presented in Figure 19 and 20 suggest that mutations in either of the regions encoding the hydrophobic domains could lead to a loss of membrane association and can lead to compromised trafficking. The addition of eGFP to the C-terminal of a hydrophobic domain mutant probably leads to compromised stability of the NS3 protein and therefore results in cleavage of the fusion protein. All four of the hydrophobic domain mutants had considerably lower levels of expression which made the detection of these proteins quite difficult. For confocal imaging the detector gain had to be increased and in the case of the eGFP-tagged proteins alternative adjustments to the brightness and contrast of images had to be made to enable comparison. This, together with the variable results that were obtained for untagged proteins, suggests that the results for these constructs should be interpreted with caution.

The NS3-PRO protein (Figure 22) also had a punctate cytoplasmic distribution but seldom any plasma membrane or nuclear membrane localisation. The NS3-CR mutant protein showed a punctate cytoplasmic distribution and localisation to the nuclear membrane as well as the presence of intranuclear bodies. In most cells the NS3-CR mutant protein showed no localisation to the plasma membrane.

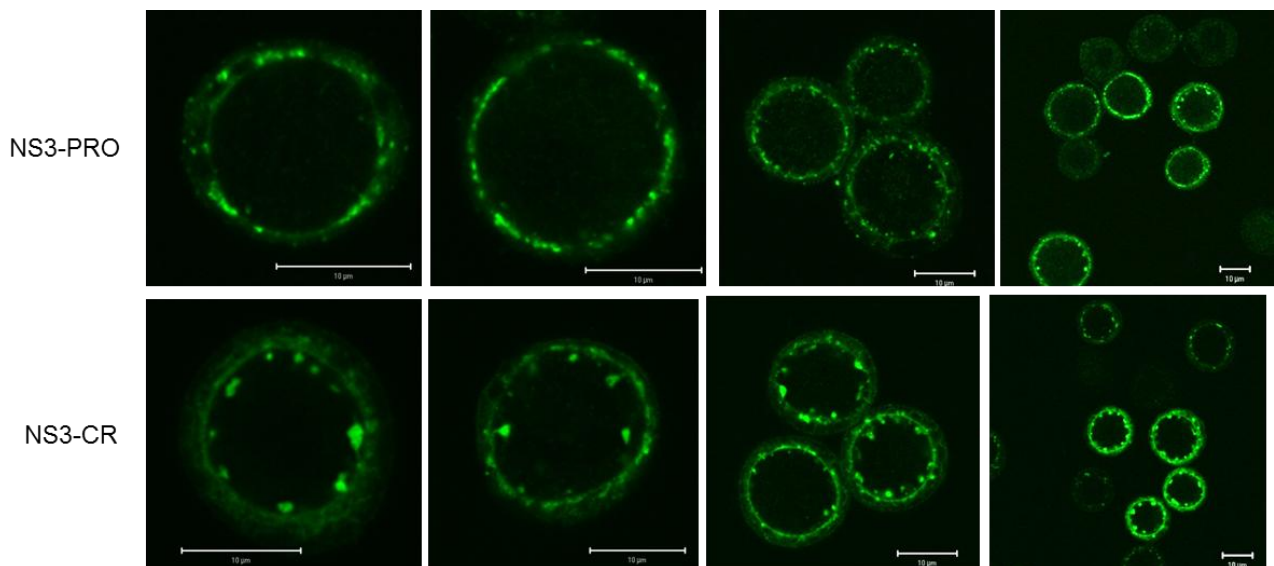


Figure 22. Confocal images (100x) of NS3 proteins that have substitutions in the proline rich region (top panel) and the conserved domain (bottom panel). Scale bars represent 10 μm.

Since both the NS3-CR and NS3-PRO mutants showed the lack of plasma membrane localisation both these regions may play an important role in enabling the migration of NS3 toward the cell membrane. Mutations in any of the conserved regions compromise the stability of the NS3 protein and leads to altered localisation patterns in insect cells. Due to the conserved nature of these regions they might carry signalling domains or interact with cellular proteins in order to migrate to the plasma membrane. In the video data, the perinuclear localisation of the NS3-PRO mutant is clearly visible whilst the NS3-CR mutant shows a strong nuclear membrane signal with INB's which appear to project into the nucleus from the nuclear membrane.

2.3.4 Subcellular fractionation analysis

From the confocal images it appeared that most of the NS3 mutant proteins showed a punctate distribution in the perinuclear cytoplasm as well as in the plasma membrane. These findings suggest that NS3 occurs most probably as an insoluble membrane associated protein inside the ER and Golgi of infected cells. In order to test this hypothesis, subcellular fractionation analysis was carried out for each construct.

Sf9 cells expressing NS3 and mutant proteins were harvested at 48 hpi and subcellular fractionation was performed. All the pellets were stored in 1 x phosphate buffered saline and the nuclear (N), particulate (P) and soluble (S) fractions of each construct were analysed on a 15% SDS-PAGE gel and detected with anti-NS3 (1/100) or anti-GFP (1/4000) antibodies on a Western blot (Figure 23).

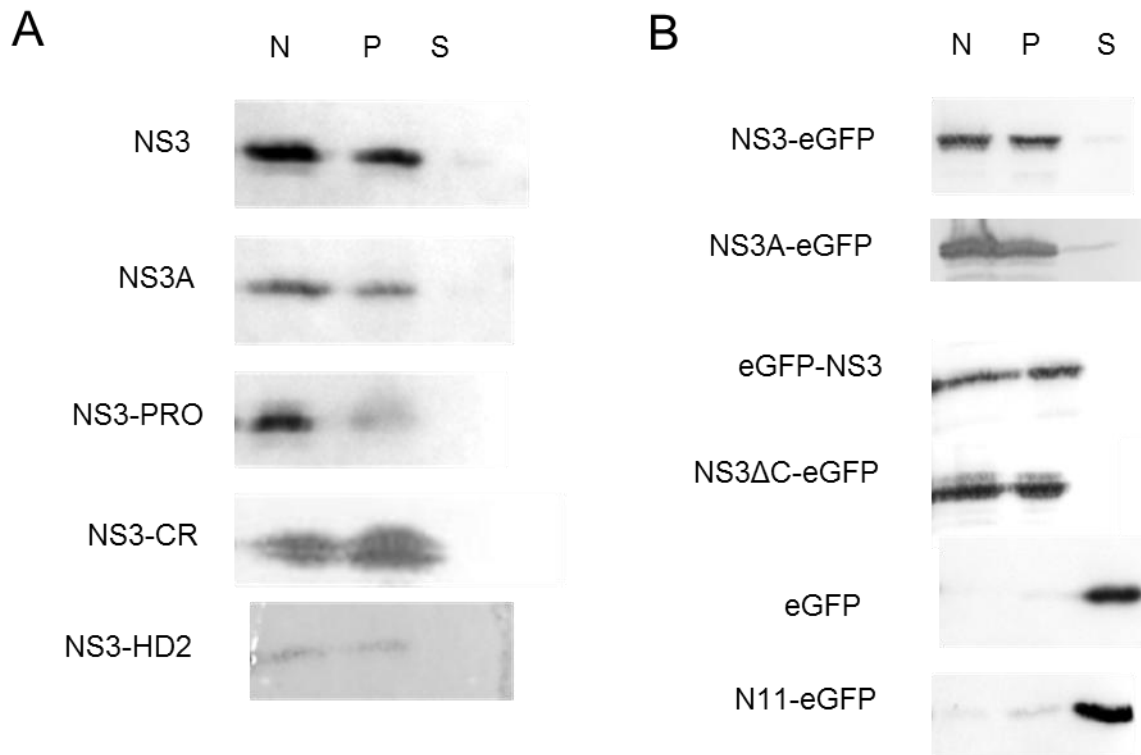


Figure 23. Western blots with anti-NS3 (A) and anti-eGFP (B) antibodies of the subcellular fractionations. Included are the nuclear fraction (N), the particulate (P) and the soluble fraction (S).

From the Western blots it can be seen that a large portion of the proteins were present in the nuclear fraction. Since the endoplasmic reticulum is closely associated with the nuclear envelope the nuclear fraction probably contains the nuclear content, the nuclear membrane as well as some of the ER and its associated proteins. The remainder of the proteins were all present in the particulate fraction which contains all other membranous organelles (vesicles or Golgi). None of the mutant proteins were detected in the soluble (cytoplasm) fraction which confirms that the NS3 protein and modified versions of NS3 were insoluble and probably membrane associated. The band intensity of the Western blots correlated with the level of expression of each protein.

The results for the NS3-eGFP, NS3A-eGFP, eGFP-NS3 and NS3ΔC-eGFP proteins were similar to the non-eGFP constructs. Almost equal amounts of these proteins were present in the nuclear and particulate fractions and a total lack of proteins in the soluble fraction. All of the wild-type eGFP protein and the bulk of the N11-eGFP protein were present in the soluble fractions, whilst a small amount of the N11-eGFP protein was also present in the particulate fraction. The intensity of the bands on the Western blot correlated with the amount of expression for each construct in Sf9 cells. As expected, the eGFP-tagged proteins showed much higher expression levels than the non-eGFP proteins.

The subcellular fractionation analysis yielded results that correspond to those found with the confocal microscopy.

2.3.5 Membrane flotation analysis

The subcellular fractionations confirmed that the wild-type eGFP and the N11-eGFP proteins were present as soluble proteins. The other NS3 constructs mainly occurred in the insoluble fraction which supports the hypothesis that these proteins are membrane associated when they are localised in the cytoplasm. To verify the results obtained with the confocal microscopy and subcellular fractionations and also indicate whether proteins that were detected in the nuclear fraction are soluble intranuclear proteins or attached to the nuclear membrane, membrane flotation analysis was performed for each construct.

Sf9 cells expressing NS3 and the mutant variants thereof were harvested 48 hpi and membrane flotation analysis was performed. For the eGFP-tagged NS3 proteins, fluorescence readings were taken. For comparative purposes, the fluorescence reading for each construct was converted to a percentage of total fluorescence (Figure 24).

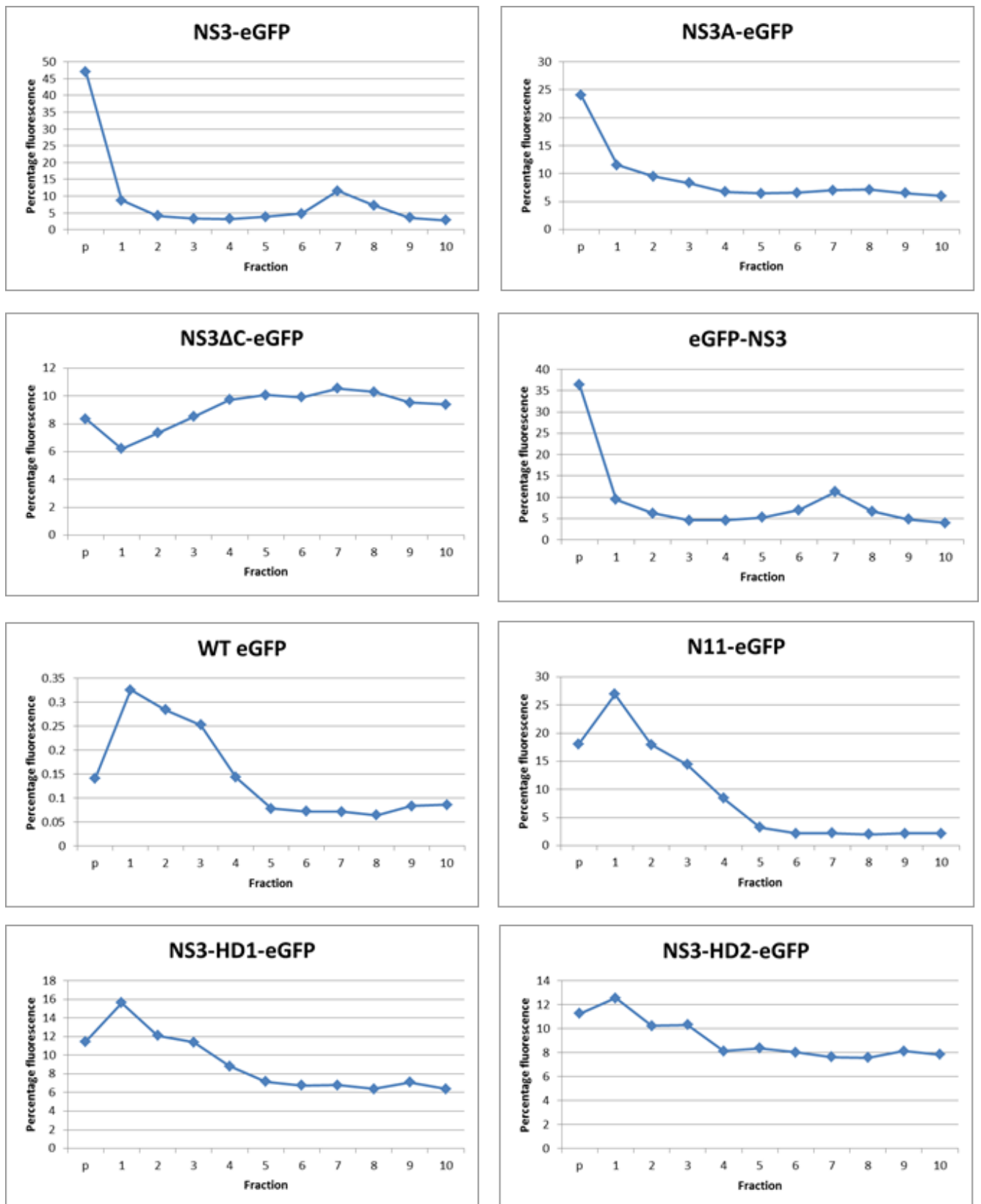


Figure 24. Fluorometric readings of eGFP fusion proteins after membrane flotation analysis. Fraction 6 – 10 defines the top of the gradient which would contain membranes and associated proteins whilst fractions 1 - 5 would contain the soluble proteins.

For each construct membrane flotation analysis was repeated three times, the average fluorescence readings are presented in the graphs. For all the constructs the bulk of the proteins

were present in the pellet fraction. This is probably a result of cells that were not lysed properly and upon centrifugation immediately spun out with the pellet. In order to enable better comparison of the proteins that were actually present in the sample that were separated in the sucrose gradient, the graphs were redrawn without the pellet fraction (Figure 25) to get an indication of whether the proteins were soluble or membrane bound.

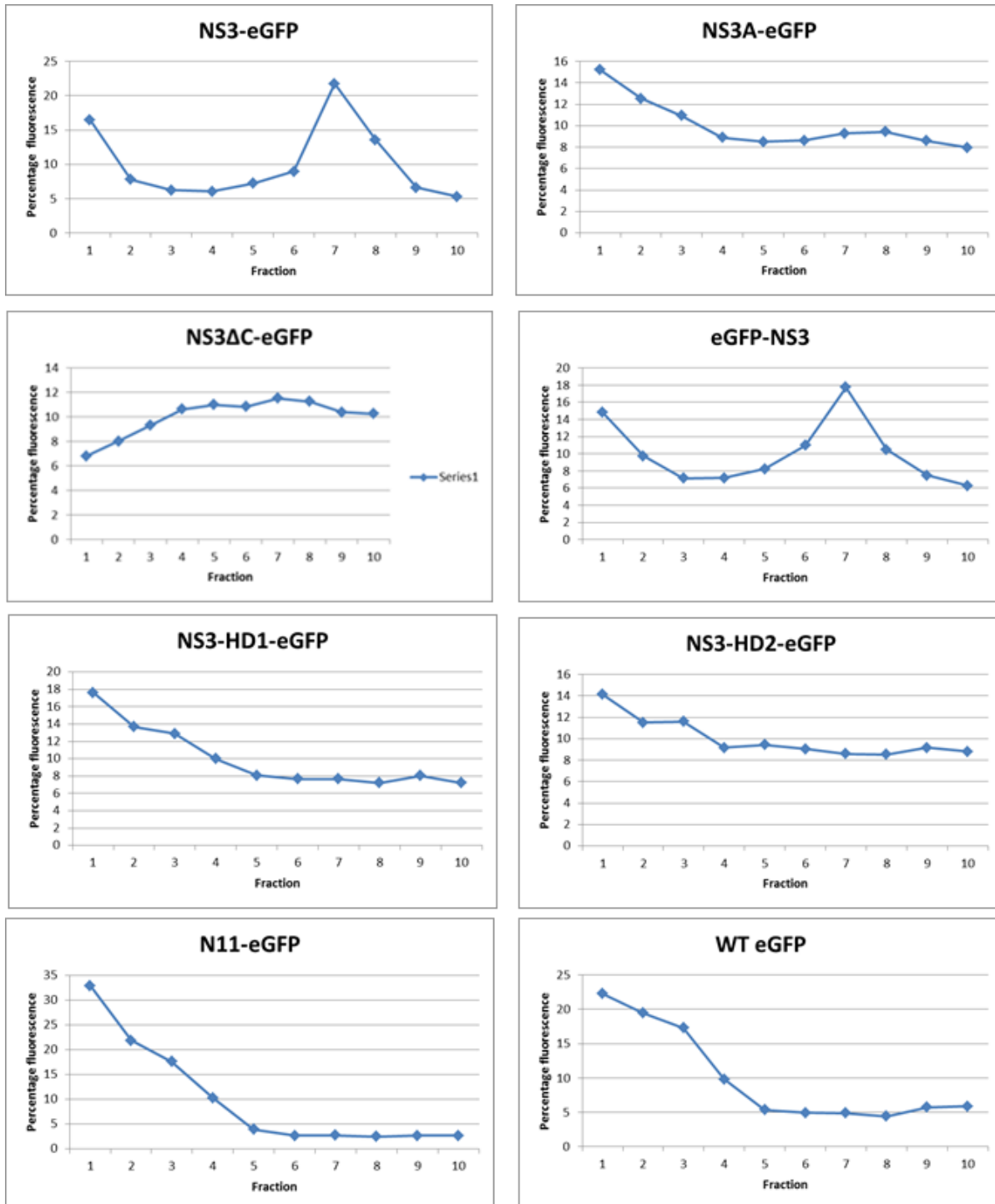


Figure 25. Fluorometric readings of GFP fusion proteins after membrane flotation analysis without the pellet fraction. Fraction 6 – 10 defines the top of the gradient which would contain membranes and associated proteins whilst fractions 1 - 5 would contain the soluble proteins.

After exclusion of the pellet fraction, it appeared that the NS3-eGFP, NS3A-eGFP and eGFP-NS3 proteins were present as both soluble and membranous proteins. The soluble component of these constructs could be attributed to the presence of the eGFP-tag since the presence of eGFP has been shown to increase the solubility of insoluble proteins (Yang *et al.*, 1996). The soluble portion of proteins could also be attributed to protein cleavage since the eGFP component could only be detected in the lower fractions as in the case of NS3-HD1-eGFP and NS3-HD2-eGFP which had very similar profiles to the WT eGFP. Both the hydrophobic domain mutants showed very low fluorescence as observed with the confocal microscopy and Western blot. Our previous confocal microscopy results have shown that very few cells were still live and viable at 48 hpi and the soluble proteins could have been caused by protein degradation from cells at this late stage during infection. As expected, practically all of the wild-type eGFP and the N11GFP proteins were present in the soluble fractions.

Following membrane flotation centrifugation and fractionation, the non-eGFP NS3 proteins were subjected to electrophoresis on a 15% SDS-PAGE gel and were detected on a Western blot with anti-NS3 (1/100) antibodies (Figure 26).

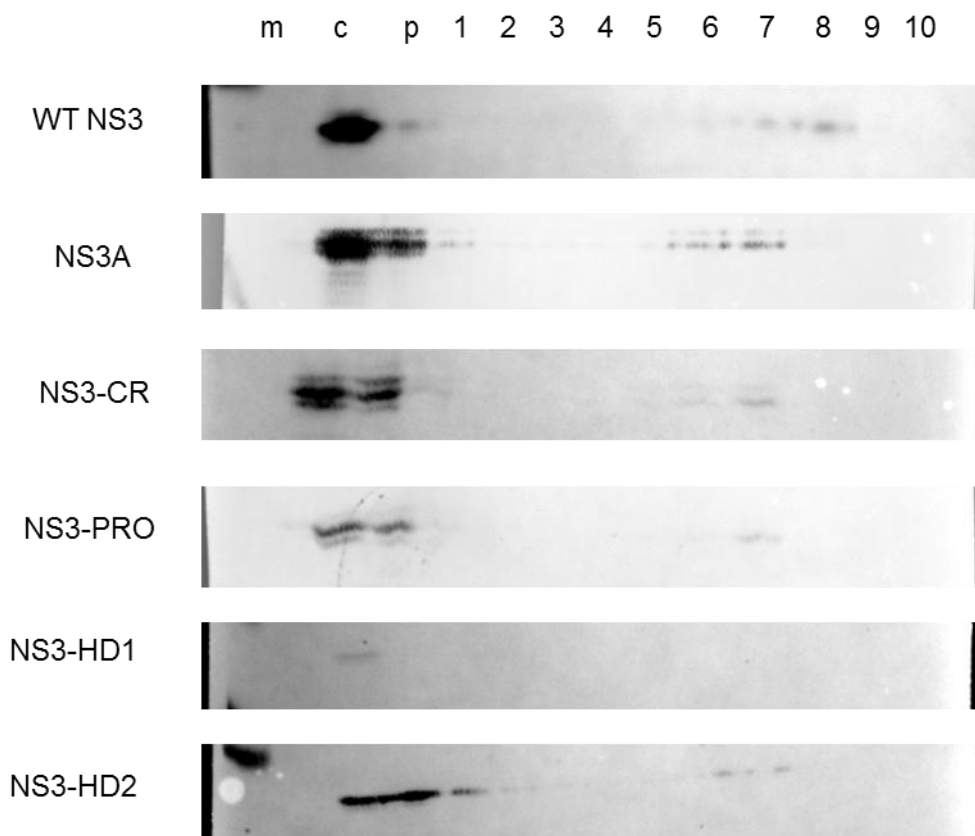


Figure 26. Western blots showing the membrane flotation analysis of the non-GFP NS3 constructs detected with anti-NS3 antibodies. An uninfected cell lysate was loaded on all gels as a negative control (m), a fraction of the cell lysate of each construct was included as control (c). Included are the pellet (p) followed by the ten (1-10) flotation fractions, where fraction 10 represents the top of the gradient.

With the exception of the NS3-HD1 protein that expressed too low for detection on a Western blot, it appeared that the bulk of the proteins were present in the pellet fraction. However if the pellet fraction is not considered and only fractions 1-10 is taken into account, then it appears that all of the non-GFP proteins have a portion of the proteins present in the upper fraction which indicate some form of membrane association. The NS3A, NS3-CR and NS3-HD2 proteins also show a faint band present in fraction 1 which indicate that a very small portion of these proteins might also occur as soluble proteins inside the host cell.

2.3.6 Localisation of NS3 in AHSV infected cells

2.3.6.1 Comparing the localisation of NS3 in insect cell expression system to AHSV infected mammalian cells

For a full analysis we compared the expression of the NS3 protein in a baculovirus expression system to NS3 expressed in AHSV infected mammalian cells. AHSV-3 infected Vero, BHK and BSR cells were fixed and immunolabeled with anti-NS3 and AlexaFluor 488 at 27 hpi. Cells were mounted for visualisation under the confocal microscope (Figure 27).

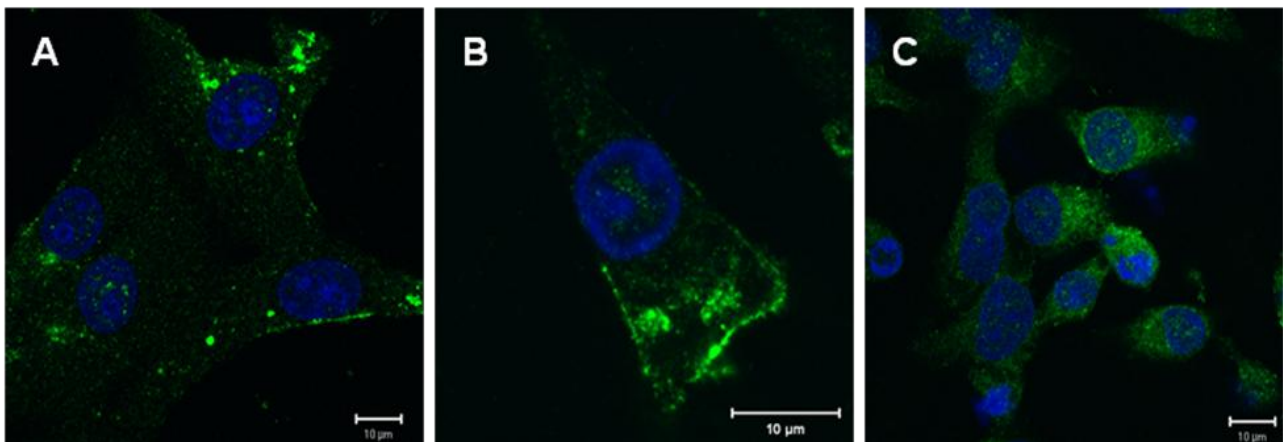


Figure 27. Confocal images (100x) showing AHSV-3 infected Vero cells at 27 hpi (A), a BHK cell at 27 hpi (B) and BSR cells (C) at 24 hpi. All cell types were fixed with methanol/acetone and immunolabeled with anti-NS3 primary antibodies (1/100) and AlexaFluor 488 (1/250) secondary antibodies. Nuclei were stained with DAPI. Scale bars represent 10 µm.

In both the Vero and BHK mammalian cells again the punctate cytoplasmic localisation pattern and plasma membrane labelling was observed, similar to the distribution in Sf9 insect cells (see Figure 16). These results suggest that similar mechanisms may exist for the trafficking of NS3 proteins in both insect and mammalian cells. In BSR cells (a BHK derivative cell line) the NS3 had a smoother appearance in the perinuclear cytoplasm and no NS3 was observed in the cell membranes. These same results were observed for all three of the biological repeats in the BSR cells.

2.3.6.2 Colocalisation of NS3 with cellular markers in baculovirus infected Sf9 insect cells expressing NS3 and AHSV-3 infected Vero cells.

Most viruses exploit host trafficking pathways in order to migrate from the point of viral assembly to the cell membrane where viruses then exit the cell either through cell lysis or viral budding. The pathway of interest in our study was the cellular secretory pathway for membranous proteins. In this pathway proteins are inserted in the lipid bilayer of a membranous organelle (such as ER) and bud from one membranous organelle to the next (ER to Golgi) until the protein reaches the target destination (plasma membrane).

All the results obtained thus far indicated that AHSV exploits the cell secretory pathway through NS3. In order to confirm this, a colocalisation study was performed with Sf9 cells infected with Bac-NS3-eGFP or Bac-NS3. The endoplasmic reticulum or the Golgi was immunolabeled with anti-calnexin (BD Biosciences) or anti-Golgin97 (Invitrogen) respectively at 27 hpi. Cells were mounted and visualised with the LSM 510 Meta confocal microscope (Figure 28 and 29).

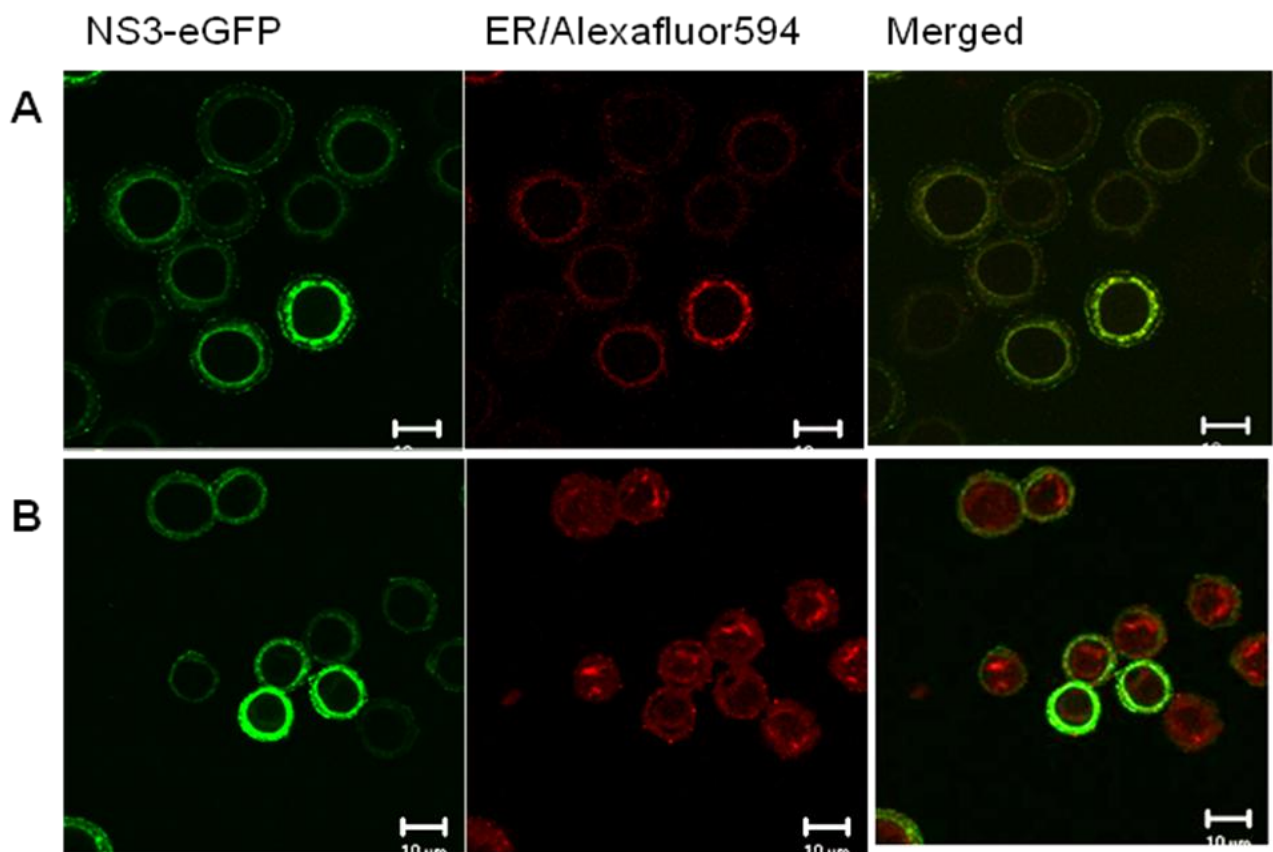


Figure 28. Bac-NS3-eGFP infected Sf9 insect cells at 27 hpi. ER was immunolabelled with anti-calnexin (A and B) primary antibodies and Alexafluor 594 (red) secondary antibodies. The scale bars represent 10 µm.

In Bac-NS3 infected Sf9 insect cells the typical punctate perinuclear cytoplasmic and plasma membrane distribution was observed for NS3 proteins. In some cells the anti-calnexin antibodies labelling was identical to the expression profile of the WT NS3 (Figure 28 A) whilst in other cells

the ER marker also labelled the nucleus non-specifically (Figure 28 B). The merged images (Figure 28 A and B, merged) which shows both signals from the red and green channels together, can be used to calculate the amount of colocalisation of different markers. The identical localisation pattern for both markers seen in Figure 28 A could be due to bleedthrough fluorescence from the green signal as a result of the overlapping excitation wavelengths of the different fluorophores.

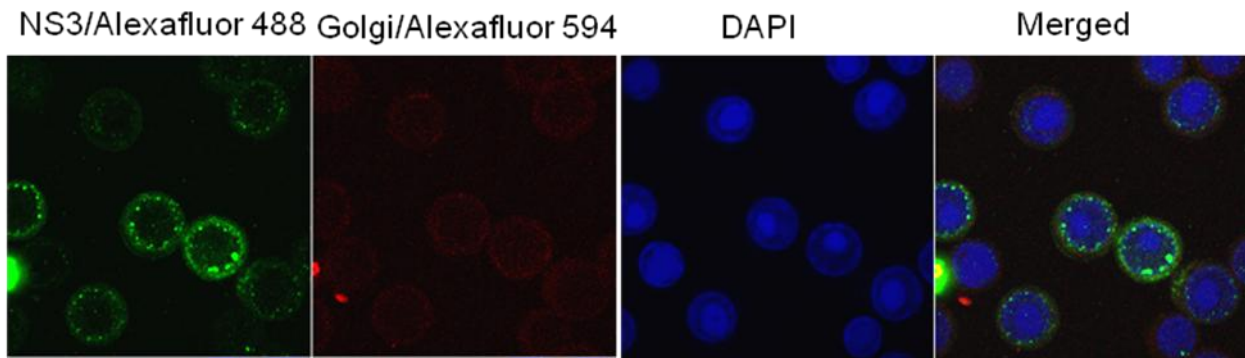


Figure 29. Bac-NS3 infected Sf9 insect cells at 27 hpi. NS3 was immunolabelled with anti-NS3 (primary antibodies) and Alexafluor488 (secondary antibodies). Golgi was immunolabelled with anti-Golgin97 primary antibodies and Alexafluor 594 (red) secondary antibodies. The nuclei were labelled with DAPI. The scale bars represent 10 μ m.

Sf9 cells that were labelled with the Golgi marker showed no or very little, non-specific labelling. It can be concluded from these results that the cellular organelle markers are probably mammalian cell specific due to the mammalian origin of the antibodies and are therefore not suitable for organelle labelling in Sf9 insect cells. The same ER/NS3 colocalisation analysis was performed on AHSV infected Vero cells. At 24 hpi cells were fixed with methanol/acetone and immunolabelled with anti-calnexin and anti-NS3 antibodies. Cells were visualised with confocal microscopy (Figure 30).

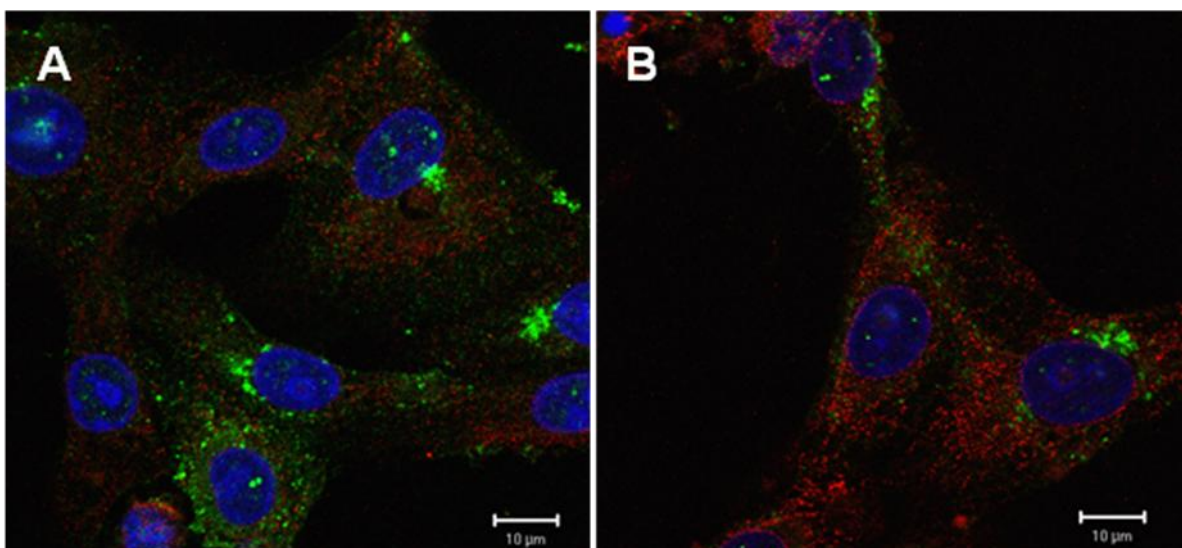


Figure 30. Confocal images of AHSV-3 infected Vero cells 24 hpi (A and B). Cells were immunolabelled with anti-NS3 primary antibodies and Alexafluor 488 (green) together with anti-calnexin (primary antibodies) and Alexafluor594 (red). The cell nuclei were stained with DAPI (blue). Scale bars represent 10 μ m.

In Vero cells the NS3 proteins again showed the punctuate perinuclear distribution. Very few cells had NS3 in the plasma membrane. The labelling of the ER marker was mainly non-specific with localisation throughout the cell cytoplasm. For this reason no conclusion could be drawn from cells double labelled with anti-NS3 and anti-calnexin antibodies. A second ER marker was therefore tested. Vero cells were infected with AHSV, and BacMam 2.0 ER marker for live cells was added to the growth medium. At 24 hpi cells were fixed with 4% PFA (as specified by the BacMam protocol) and upon permeabilisation, cells were immunolabelled with anti-NS3 antibodies. Cells were mounted and visualised with the confocal microscope (Figure 31). I expected to observe a red labelling pattern in the perinuclear cytoplasm from approximately 16 hours after addition of the BacMam 2.0 marker to the cell medium.

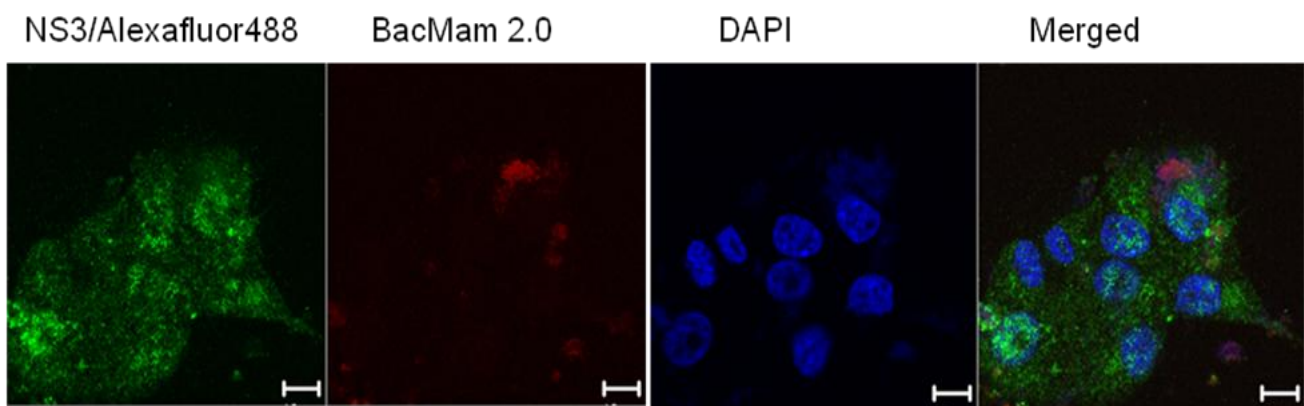


Figure 31. Confocal images of AHSV-3 and BacMam 2.0 infected Vero cells at 24 hpi. Cells were immunolabelled with anti-NS3 primary antibodies and Alexafluor 488 (green). Cell nuclei were stained with DAPI (blue). Scale bars represent 10 µm.

The BacMam 2.0 ER marker also failed to label the ER in Vero cells. The labelling of the NS3 proteins was distorted as well. NS3 was observed throughout the cell cytoplasm and also as granules in the cell nuclei. In order to optimise and eliminate all factors that could possibly influence the ER labelling, experiments were repeated without the presence of the AHSV, in a live cell chamber to determine if the BacMam expression system worked. All of the experiment yielded similar results as previously discussed. Since it was possible that this live cell marker could also be cell type dependent, the experiment was repeated in BSR and BHK mammalian cells. The results, however, remained inconclusive.

To determine whether NS3 colocalises with the Golgi apparatus, Vero cells were infected with AHSV. Cells were fixed with methanol/acetone at 24 hpi and immunolabelled with anti-NS3 and anti-Golgin97 antibodies for NS3 proteins and the Golgi respectively (Figure 32). Similar to the ER marker, I expected that the red labelled Golgi marker would have a perinuclear distribution.

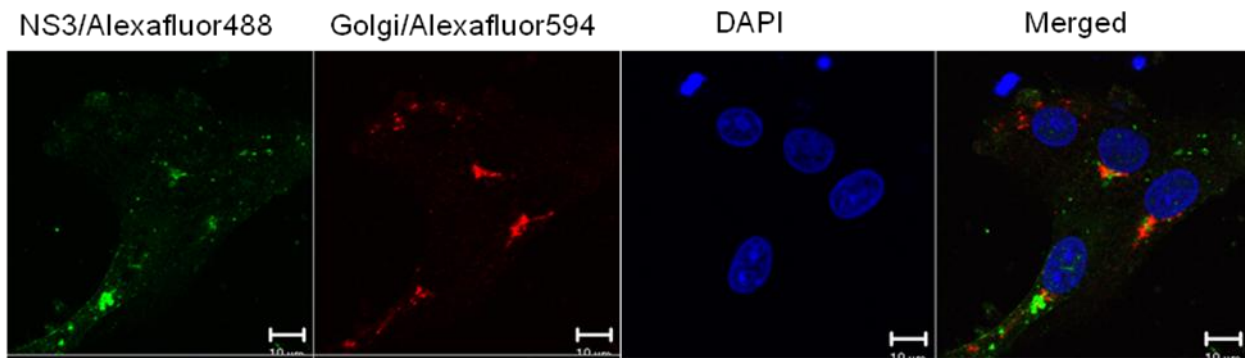


Figure 32. Confocal images of AHSV-3 infected Vero cells at 24 hpi. Cells were immunolabelled with anti-NS3 primary antibodies and Alexafluor 488 (green). Golgi was immunolabelled with anti-Golgin97 primary antibodies and Alexafluor 594 (red) secondary antibodies. Cell nuclei were stained with DAPI (blue). Scale bars represent 10 µm.

The Golgi marker was observed in the perinuclear cytoplasm in close proximity to the cell nucleus as was expected. From the merged images it also appeared that the NS3 proteins colocalised with the Golgi marker. The quantification and colocalisation of the Golgi marker with the NS3 proteins will be discussed in more detail in section 2.3.6.3.

It was decided that two alternative cellular cytoskeletal markers would be tested in order to determine if NS3 interacts with the cell cytoskeletal proteins in order to migrate to the plasma membrane. Firstly colocalisation was performed with anti-tubulin which labels microtubules, in order to see if AHSV NS3 uses microtubules for viral egress similar to poxvirus, herpes virus and HIV (Ward, 2011). BSR cells were infected with AHSV-3, fixed with methanol/acetone at 24 hpi and immunolabelled with anti-NS3 and anti-tubulin before visualisation with confocal microscopy (Figure 33).

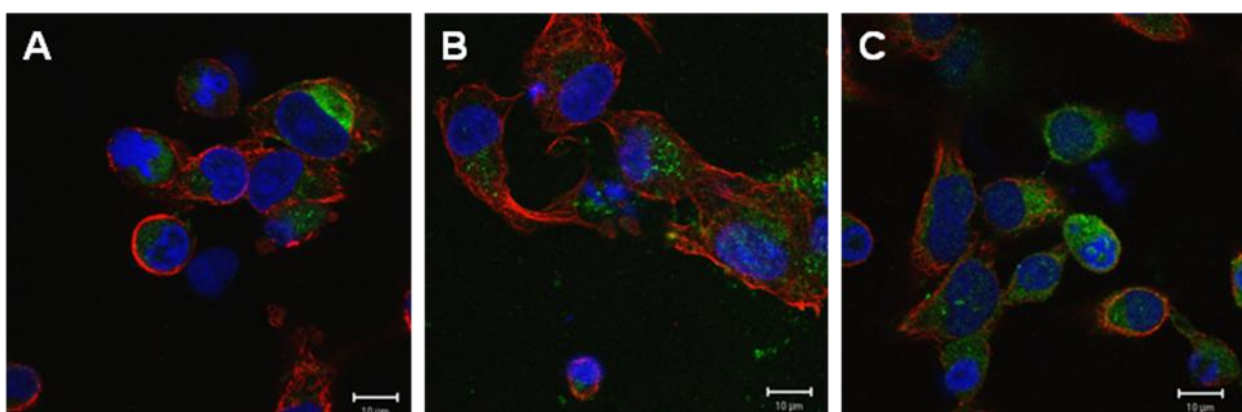


Figure 33. Confocal images of AHSV-3 infected BSR cells at 24 hpi (A, B and C). Cells were immunolabelled with anti-NS3 primary antibodies and Alexafluor 488 (green) as well as anti-tubulin primary antibodies and Alexafluor 594 (red) secondary antibodies. Cell nuclei were stained with DAPI (blue). Scale bars represent 10 µm.

In all the BSR cells the labelling of the microtubules was very specific. In all the cells it was clear that there was no colocalisation between NS3 and the microtubules. The microtubule labelling had

no interference with the localisation of the NS3 proteins which was observed to have a punctuate perinuclear localisation pattern. No NS3 was observed in the plasma membrane. These results confirmed that NS3 does not interact with microtubules in order to migrate to the plasma membrane.

The second cytoskeletal marker that was tested was vimentin. It was previously shown that BTV VP2 interacts with vimentin in order to establish viral egress (Bhattacharya *et al* 2007). Since VP2 has also been shown to interact with NS3 (Beaton, *et al* 2002) it was then expected that there should be some colocalisation between NS3 and vimentin. BSR mammalian cells infected with AHSV-3 were fixed (methanol/acetone) and immunolabelled with anti-NS3 and anti-vimentin antibodies. Slides were mounted and visualised with a confocal microscope (Figure 34).

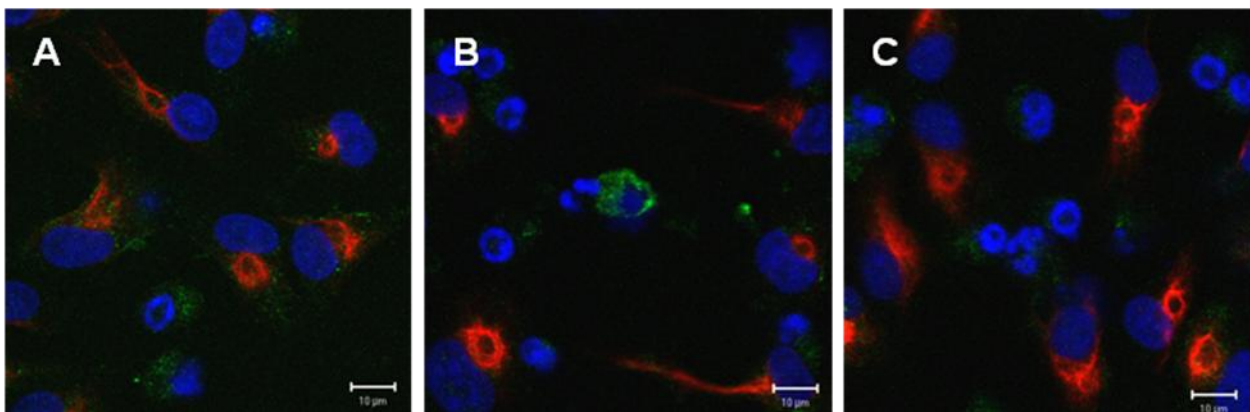


Figure 34. Confocal images of AHSV-3 infected Vero cells at 24 hpi (A,B and C). Cells were immunolabelled with anti-NS3 primary antibodies and Alexafluor 488 (green) as well as anti-vimentin and Alexafluor 594 (red) secondary antibodies. Cell nuclei were stained with DAPI (blue). Scale bars represent 10 µm.

The vimentin labelling was as expected in the juxtannuclear cytoplasm. It seemed, however, that on a technical level the labelling of the vimentin interfered with the labelling of the NS3 antibodies, since the green labelling was only observed in cells that had no labelling of the vimentin (Figure 34 B). This was unfortunate since no conclusions could be drawn from the analysis.

Colocalisation experiments performed with the Golgi marker will be used in the next section to explain the colocalisation analysis using the ImageJ software.

2.3.6.3 Quantification of NS3 colocalisation with the Golgi apparatus using ImageJ™

For quantification of the colocalisation of NS3 proteins with the Golgi marker we used ImageJ™ software. In order to do statistical analysis of the images, fluorescent signals which were presented as computer pixels had to be converted to data value points. Firstly, the combined image was split

into the three different colour channels, of which each was converted to an 8-bit grayscale image (Figure 34).

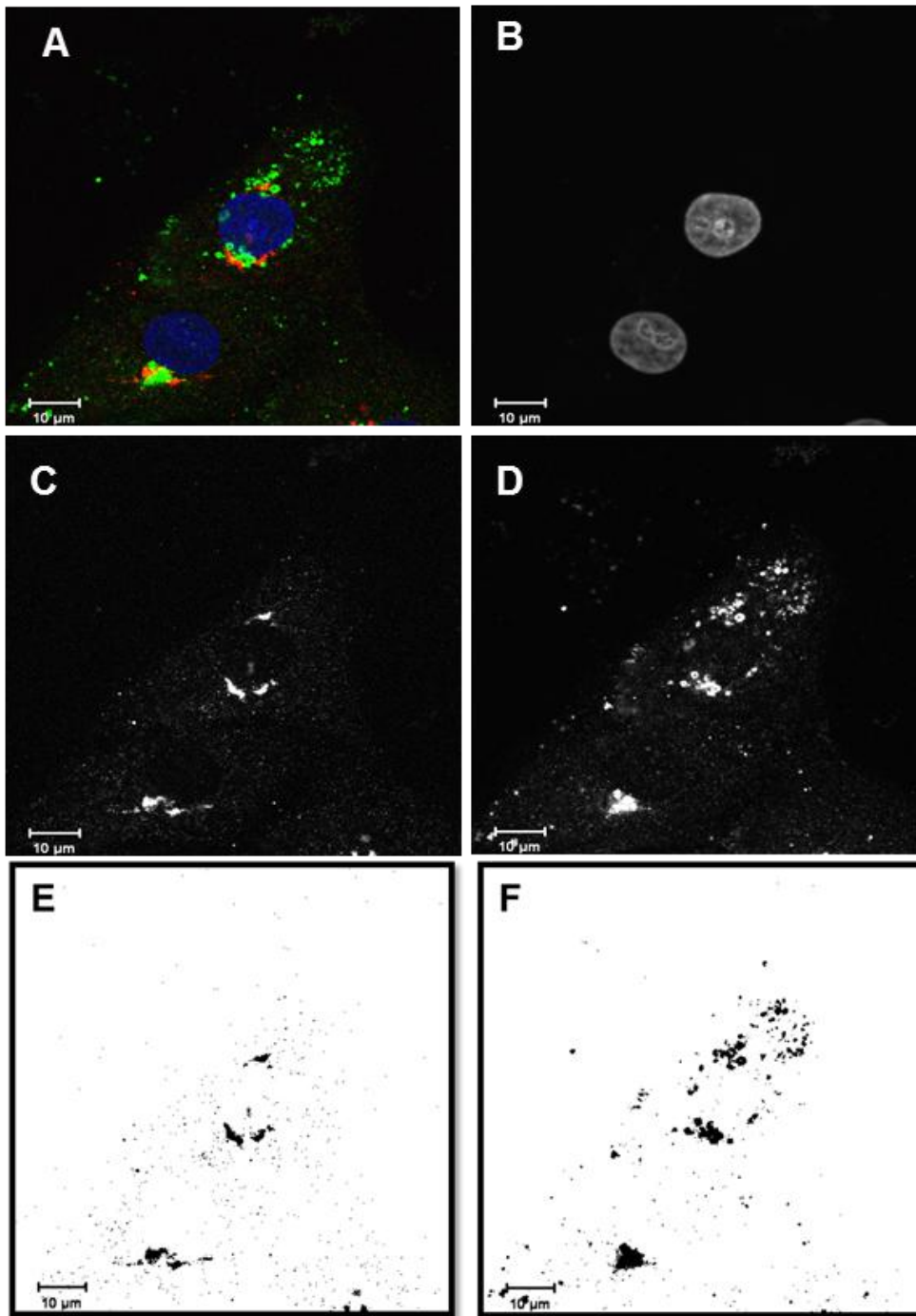


Figure 34. For image analysis a merged image (A) is split into blue (B), red (C) and green (D) channels and converted to 8-bit gray-scale images for quantification. Images E and F represent high intensity fluorescent pixels (black) for red (E) and green (F) channels. Scale bars represent 10 μm .

The DAPI stained images (Figure 34 B) were discarded from the analysis at this point since we were only interested in the red and green colocalisation. Images were scaled and a threshold value for each channel was determined according to the fluorescence intensity. Once the threshold

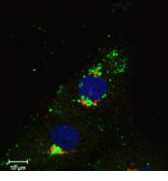
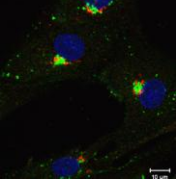
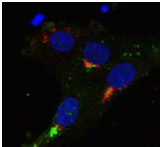
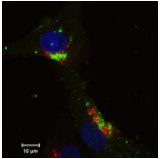
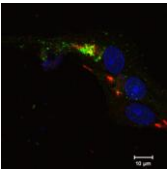
values were determined the images were converted to black and white images which include only the high intensity fluorescent pixels (Figure 34 E and F).

Using the red channel as reference, specific regions of interest (ROI) were identified which would then represent the Golgi apparatus. Using the ImageJ™ ROI manager, the number of pixels in each ROI was calculated. The software then allowed the user to specify the same ROI on the green channel and in the same way, the number of pixels from the green channel in each ROI could be calculated. Next the colocalisation finder was used to determine the colocalised pixels from both channels. Again the ROI manager was used to calculate the number of pixels that colocalise between the two channels. This value was divided by the value obtained for the red channel to determine the percentage of colocalisation for each ROI in each of the images. Five images were selected at random, and for each a number of ROIs were identified for analysis.

Alternatively we also determined the Manders' coefficients for each image that was analysed, using the relevant ImageJ™ plugin (Table 6). This plugin calculates the amount of colocalisation for the whole image and not just a single defined area. The Manders' coefficient is a statistical parameter that can be interpreted as the amount of true colocalisation between two different channels and is frequently used for application to biological systems since it takes into account that some stains/antibodies has higher affinity for binding to their targets than others. A Manders' coefficient value of one indicates absolute colocalisation whilst a value of zero is the total absence of colocalisation.

For each of the images the Manders' coefficient was higher than the calculated percentage of colocalisation. This could have been because for the Manders' calculation, the software takes each pixel in the red channel and compared it to each of the pixels in the green channel for the whole image to determine if there is any colocalisation. Since there were no threshold constraints for this plugin, the Manders' coefficient was expected to be higher than the true amount of colocalisation. The partial colocalisation that was observed between the NS3 proteins and the Golgi was expected since the NS3 proteins traffic through the Golgi to the plasma membrane. The images and colocalisation data are summarised in Table 6.

Table 6. A summary of the co-localisation analysis performed with ImageJ™ software. The red channel represents the Golgi apparatus labelling and the green channel represents NS3.

Image name:	Channel	Threshold		area 1	area 2	area 3	area 4
Vero Golgi 1 	Red	73		506	442	136	
	Green	68		802	587	280	
	Co-localized points			104	56	38	
	Percentage co-localisation			20.55	12.67	27.94	
	Manders' coefficient		0.56				
Vero Golgi 2 	Red	82		175	495	470	398
	Green	83		103	731	776	576
	Co-localized points	171-255		37	104	124	59
	Percentage co-localisation			21.14	21.01	26.38	14.82
	Manders' coefficient		0.56				
Vero Golgi 3 	Co-localized points	157-255		72	96	102	1
	Percentage co-localisation			33.18	14.75	18.96	0.55
	Manders' coefficient		0.58				
Vero Golgi 4 	Red	87		1433	727		
	Green	80		1064	946		
	Co-localized points	152-255		593	301		
	Percentage co-localisation			41.38	41.40		
Vero Golgi 5 	Red	85		904			
	Green	78		1221			
	Co-localized points	172-255		467			
	Percentage co-localisation			51.66			
	Manders' coefficient		0.68				

The variable percentage of colocalisation that we calculated for our samples could be expected if NS3 only migrates through the ER and the Golgi apparatus upon trafficking toward the plasma membrane.

As we had established that AHSV NS3 protein utilizes the cellular secretory pathway for membrane proteins we wanted to see the effect of a chemical inhibitor on the migration and localisation of NS3 in Sf9 and Vero cells.

2.3.6.4 Pathway inhibition with Brefeldin A

The results obtained from the colocalisation studies support the hypothesis that NS3 proteins migrate through the ER to the Golgi and then to the plasma membrane. To further test this theory blockage of a pathway was performed by means of chemical inhibition. Brefeldin A (BFA) is a fungal metabolite produced by *Penicillium brefeldianum*. BFA blocks the secretory pathway by disrupting the movement of proteins from the ER to the Golgi apparatus in mammalian cells.

For this experiment, Sf9 cells were grown on coverslips and infected with Bac-NS3-eGFP or Bac-NS3. From 16 hpi the culture medium was constantly supplemented with freshly prepared BFA. At 27 hpi cells were fixed and immunolabelled in the case of Bac-NS3. Coverslips were mounted onto microscope slides and visualised with the LSM Meta 510 confocal microscope (Figure 35).

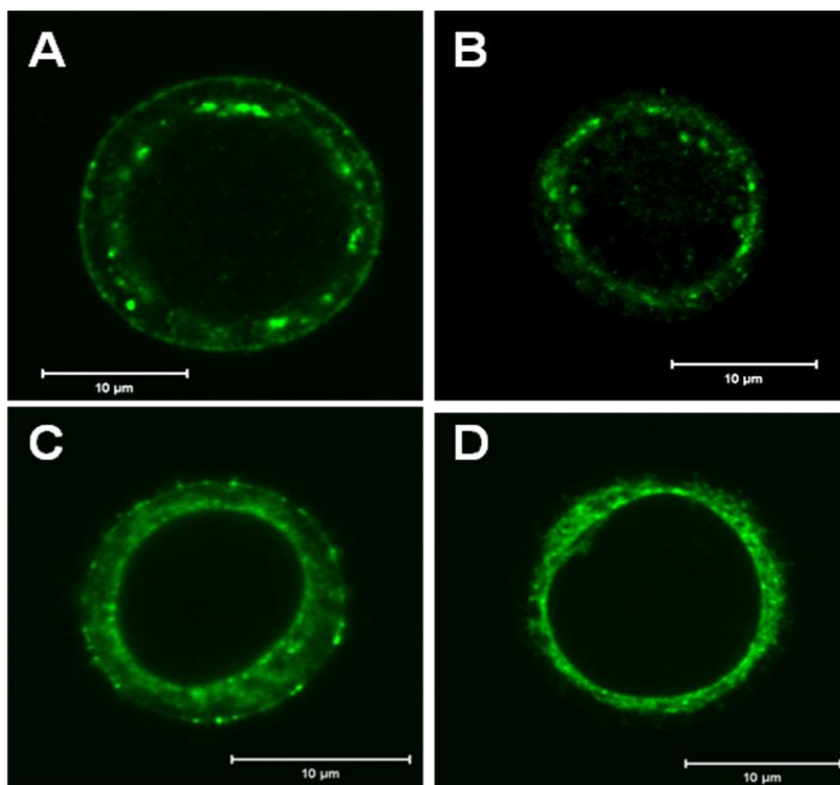


Figure 35. Bac-NS3 (A and B) and Bac-NS3-eGFP (C and D) infected Sf9 insect cells. Figure A and C represent control cells and B and D show infected cells after treatment with BFA. Scale bars represent 10 µm.

In the control cells (Figure 35 A and C) the typical punctate perinuclear and plasma membrane distribution of the NS3 proteins were observed. After treatment with the chemical inhibitor BFA, the punctate perinuclear localisation (putatively ER) was maintained in the cells but approximately ninety percent of the cells failed to have any NS3 localising to the plasma membrane. These results support the hypothesis that NS3 proteins exploit the host cell secretory pathway, since blocking the ER to Golgi trafficking the NS3 proteins were prevented from migrating to the plasma membrane.

To determine whether the NS3 trafficking mechanism is the same for mammalian and insect cells, Vero cells were grown on coverslip and infected with AHSV. From approximately 2 hpi cells were treated with BFA. At 27 hpi Vero cells were fixed and immunolabeled with anti-NS3 and anti-Golgin97 primary antibodies and Alexafluor 488 and Alexafluor 594 secondary antibodies. Cells were mounted and visualised with the LSM 510 Meta confocal microscope (Figure 36).

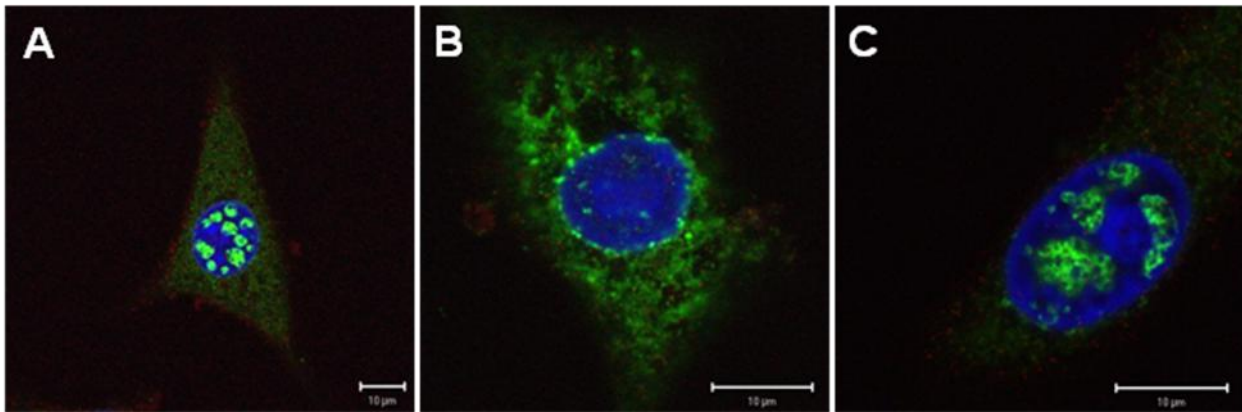


Figure 36. AHSV infected Vero cells after treatment with BFA (A, B and C). Cells were labelled with anti NS3 and anti-Golgin97 at 27 hpi. Nuclei were stained with DAPI. Scale bars represent 10 μ m.

Upon treatment of cells with BFA, it was observed that all Golgi labeling was lost which could be a result of the specific target protein (Golgin97) not reaching the Golgi apparatus due to the inhibition of ER to Golgi transport. The normal NS3 localisation and trafficking was also altered. Some cells showed a smooth localisation pattern of NS3 throughout the cytoplasm whilst in other cells the NS3 localised to the nuclear membrane and formed intranuclear bodies. These intranuclear clumps of protein did not appear to be associated with the membranes. No cells were observed to have any NS3 in the plasma membrane indicating the NS3 protein also utilizes the cell secretory pathway for membrane bound proteins for trafficking to the plasma membrane in Vero cells.

2.4 DISCUSSION

The BTV nonstructural proteins NS3/A have been well characterised and classified in terms of the role they play in virus morphogenesis, host cell pathogenicity and cytotoxicity. A number of highly conserved regions in AHSV NS3 have been identified based on sequence comparison between different AHSV strains. To date, the exact role that AHSV NS3 and NS3A play in virus trafficking and the contribution of each of the conserved regions in this regard remains an unanswered question.

The primary aim of this project was to look into the localisation and trafficking of AHSV-3 NS3 and NS3A. In order to do this, previously constructed NS3 mutants were compared to wild-type NS3 proteins to resolve some of the questions regarding the contribution of the conserved domains toward protein localisation and trafficking. We also considered the use of eGFP as a reporter molecule and considered the localisation of eight previously constructed NS3-eGFP fusion proteins. Thirdly the localisation of NS3 was compared in Sf9 insect cells and mammalian Vero cells to determine whether there are any differences between the mechanisms of trafficking and localisation between NS3 expressed in a baculovirus expression system and AHSV infected Vero cells. Subsequently, pathway inhibition was performed by addition of a chemical inhibitor, Brefeldin A, to both the insect and mammalian cells upon infection with NS3 or AHSV-3 respectively. This was done in order to determine if NS3 uses the host cell secretory pathway for membrane proteins to establish trafficking to the plasma membrane. Localisation and trafficking of proteins were mainly assayed by means of confocal microscopy and then verified with membrane association assays. To enable us to address the specific aims we had fourteen different NS3 constructs available as recombinant baculoviruses for expression in Sf9 insect cells. Before we were able to start with the comparative analysis a number of procedures had to be optimised for our purposes.

Chemical fixing of cells can greatly influence intracellular antigen binding, since it can lead to epitope destruction, loss or leaching, unmasking or redistribution of antigens (Schimenti, Jacobberges, 1992). Chemical fixatives fix cells by either coagulating and cross-linking of proteins (Pawley, 2006). In the literature there is a wide range of methods available for cellular fixation prior to immunolabeling. Numerous studies have tried to optimise and compare different chemical fixatives (Gaasbeek Janzen *et al.*, 1984; Gerna *et al.*, 1992; Pawley, 2006; Schimenti, Jacobberges, 1992) and the only consensus in the literature is that the fixative of choice depends largely on the cell type and the antigen or protein to be studied (Belitsky *et al.*, 2002; Clevenger *et al.*, 1985; Kurki *et al.*, 1988; Mann *et al.*, 1987; Pawley, 2006). It was therefore decided that selected methods should be optimised for our study. These included fixing with 4% paraformaldehyde and additional treatment with Triton X-100 for membrane permeabilisation (during immunolabeling) or alternatively fixing with ice cold 1:1 methanol/acetone. Methanol/acetone fixes cells by dehydration which leads to protein coagulation. This is a popular

fixative since it is easy to apply, has a high reproducibility and preserves antigen recognition sites (Pawley, 2006). The drawback of methanol/acetone fixation is that due to the dehydration it can lead to severe cell shrinkage which can influence 3-D imaging analysis with confocal microscopy. Paraformaldehyde fixation causes cross-linking of proteins by forming methylene bridges between reactive groups (Pawley, 2006). The cross-linking of proteins can influence antigen binding sites and subsequently antibody binding during immunolabeling. A solution of 4% PFA has been described as a useful fixative for studying membrane bound proteins, however the lack of solubility in water can lead to high variability in the application of this fixative (Pawley, 2006).

Upon comparison of Bac-NS3-eGFP and Bac-NS3 infected Sf9 cells exposed to different chemical fixatives it was found that all fixatives yielded similar quality results for both proteins. Neither of the fixatives had any influence on antibody binding during the immunolabelling (the Bac-NS3 infected cells were immunolabelled with anti-NS3 antibodies and Alexafluor™488) however a significant amount of background labeling was observed in the control cells upon fixation with 4% PFA and permeabilisation with Triton X-100 (Figure 14). Even though it is known that aldehyde fixatives can cause autofluorescence (Hibbs, 2004) there are numerous studies that have made use of this fixative for studying the trafficking of viral proteins with the use of confocal microscopy (Jo *et al.*, 2010; Johnson *et al.*, 2010; Li *et al.*, 2010; Wang *et al.*, 2010). Immunolabeled Sf9 cells that were fixed with 4% PFA also presented with a grainy appearance when compared to the methanol/acetone fixed cells. The grainy appearance could be an artifact of the cross-linking by the PFA. For the above mentioned reasons, 50% (v/v) methanol/acetone was the preferred method for application in our study.

For further optimisation we conducted a time trial to establish the optimal time post infection for confocal analysis of the NS3 proteins. Expression under control of a polyhedrin promoter only starts 18 hours post infection. Naturally AHSV infected insect (*Culicoides*) host cells show little or no cell damage (Van Staden *et al.*, 1995) but expression of AHSV NS3 proteins in Sf9 cells as recombinant baculoviruses have severe cytotoxic effects after 48 hours of infection (Van Niekerk *et al.*, 2001a). For the time trial, Sf9 insect cells were infected with Bac-NS3-eGFP and analysed at 18, 24, 27, 30, 36, 42 and 48 hpi. At 18 hpi the majority of the cells showed low levels of expression which was expected due to the late onset of expression under control of the polyhedrin promoter. Cells at 24, 27, 30, 36 and 42 hpi presented similar fluorescence signals whilst at 48 hpi only few cells were present in the sample and there were variable fluorescence patterns ranging from very low to very high. The cells that showed good fluorescence at 48 hpi were probably due to secondary infection and were therefore at an earlier stage of infection. After considering the results it was decided that all further confocal analysis would be carried out at 27 hpi.

AHSV NS3 is the second most variable of the 10 AHSV proteins that are produced (Van Niekerk *et al.*, 2001b). Van Staden *et al.* (1991) have identified a number of conserved regions in this highly variable protein which suggest a functional role. NS3 has been shown to localise to sites of viral release (plasma membrane) in both insect and mammalian cells (Stoltz *et al.*, 1996). A previous study by van Niekerk *et al.* (2001b) aimed to identify possible roles for the conserved regions of AHSV NS3 protein. Five mutant proteins were constructed and expressed as recombinant baculoviruses in Sf9 insect cells. These mutants were designated NS3A (NS3 lacking the first eleven N-terminal aa), NS3-PRO (proline rich region or L-domain mutant), NS3-CR (conserved region mutated), NS3-HD1 (first hydrophobic domain mutated) and NS3-HD2 (second hydrophobic domain mutated). Cell viability assays revealed that the NS3A, NS3-PRO and NS3-CR proteins had the same cytotoxic effects on infected cells as the wild-type NS3. The study suggested that these conserved regions played no role in signaling of NS3 to membrane targets since the mutations did not influence the integral membrane status of the proteins. The NS3-HD1 and NS3-HD2 proteins had no cytopathic effect on infected cells. The study concluded that mutations in either of the hydrophobic membrane spanning domains abolished the proteins ability to insert correctly into the lipid bilayer and therefore these proteins fail to reach the plasma membrane (Van Niekerk *et al.*, 2001a).

Sequence analysis revealed that in the NS3 protein available to us, the serine had been substituted with a proline at position 602. Since the NS3 protein is the second most variable protein of AHSV one would not suspect that this substitution should necessarily affect the protein function. The mutant, however, included a ring structure which could affect secondary protein folding and as a result could possibly influence the protein trafficking and interactions with host cell proteins. Therefore the NS3A gene was amplified and cloned from a pUC-S10 vector (which contains the full-length NS3 gene) with NS3A specific primers using the TOPO cloning kit. For some of the mutant proteins we also had eGFP-tagged versions available to us. These mutants were considered together with their non-eGFP counterparts to determine whether eGFP can be used as a suitable reporter molecule for studying AHSV NS3 trafficking and localisation.

Upon analysis with a laser scanning confocal microscope we found that the wild-type immunolabeled NS3 protein had a punctate perinuclear distribution as well as localisation to the nuclear and plasma membrane. This finding correlated with previous studies also conducted on AHSV NS3 localisation (Stoltz *et al.*, 1996; Van Staden *et al.*, 1995). The NS3-eGFP and eGFP-NS3 proteins showed identical localisation profiles to that of WT NS3. Both the NS3-eGFP and eGFP-NS3 proteins were, however, expressed at much higher levels (as confirmed by SDS-PAGE and Western blot) and also had much higher fluorescence signals under the confocal microscope. Considering the fractionation results, all three full-length NS3 variants presented with identical bands in the nuclear and particulate fractions. The nuclear fraction can contain all proteins

associated with the nuclear membrane as well as ER associated proteins since the ER membrane is continuous with the nuclear membrane. The particulate fraction contains all the membranous organelles and since NS3 is a membrane protein this result was expected. Membrane flotation analysis confirmed all the previous confocal and fractionation results that all three the full-length NS3 variants were membrane associated. NS3-eGFP and eGFP-NS3 also had a soluble component which could be attributed to the addition of the eGFP tag which can increase the solubility of proteins (Yang *et al.*, 1996).

The NS3A mutant which lacks the first 11 amino acids of the full length NS3 protein showed mixed localisation profiles. In approximately 20% of cells the distribution was indistinguishable from the wild-type NS3 localisation, whilst in 80% of the population of cells intranuclear bodies (INBs) were present in the nuclei. These INBs appeared to be in close proximity to the nuclear membrane suggesting that they could be membrane associated. In the cells that had the intranuclear bodies present, little or no plasma membrane localisation was observed again suggesting that a mutation compromising normal trafficking signaling could result in the formation of INBs (possibly from normal baculovirus trafficking). In the NS3A-eGFP infected cells, the localisation was indistinguishable from the WT NS3 with punctate perinuclear, nuclear and plasma membrane distribution. The NS3A-eGFP fusion protein was cloned from the same NS3A that we found to contain the S602P mutation and could therefore also contain the mutation. The expression levels of NS3A-eGFP were higher than the NS3A protein but were lower than those of NS3-eGFP and eGFP-NS3 (as confirmed by Western blot). Apart from the confocal analysis, the subcellular fractionation and membrane flotation analyses for NS3A and NS3A-eGFP yielded results indistinguishable from that obtained for WT NS3 in which the proteins are mainly observed in fractions that represent membranes. The NS3A-eGFP was also observed in the soluble fractions similar to the NS3-eGFP and eGFP-NS3 proteins. Through the results that were obtained, the role of NS3A and the reason why this protein is naturally produced in host cells remains unanswered.

In bluetongue, the prototype virus of the orbivirus group, the NS3 protein has been shown to interact with the annexin II complex, a host cell protein responsible for exocytosis. Yeast two-hybrid analysis revealed that the N-terminal of NS3 interacts with a p11 protein (calpactin light-chain which interacts with a p36 protein to form the annexin II complex) whilst the C-terminal of NS3 interacts with VP2 (on the outer capsid of newly formed virus particles) to establish trafficking of the virions to the plasma membrane, where viral release takes place (Beaton *et al.*, 2002). Celma and Roy (2011) established that only the full-length BTV NS3 protein is able to interact with p11 whilst NS3A fails to colocalise or interact with p11 (Celma, Roy, 2011). This indicates that the trafficking mechanisms for the NS3 and NS3A proteins differ and could therefore explain the differences in the localisation that was observed for the non-eGFP proteins in my study. By using reverse genetics and a virus rescue experiment it was also observed that BSR cells expressing

only an NS3A mutant failed to produce any plaques, however virus recovery was observed after addition of NS3. These results suggest that NS3A might possibly play a role in virus replication instead of trafficking. With the recent development of a reverse genetics system for AHSV (Matsuo *et al.*, 2010) it might now be possible to resolve the exact role of AHSV NS3A proteins in the viral life cycle.

Many viruses encode late domain motifs (L-domains) which interact with host cell ESCRT proteins to enable viral trafficking through the vacuolar sorting pathway (Chen, Lamb, 2008). In this pathway viruses have been shown to use budding as one of the common modes of viral release. In BTV it was found that the L-domain of NS3 interacts with the Tsg-101 protein and thereby hijacks the vacuolar protein sorting (Vps) pathway to establish viral trafficking to the plasma membrane and release through budding (Wirblich *et al.*, 2006). Confocal analysis of the NS3-PRO (L-domain mutant) protein revealed that this mutant seldom localise to the plasma membrane and was never observed in the nuclear membrane. The NS3-PRO mutant protein had a punctate localisation in the perinuclear cytoplasm. With the subcellular fractionation analysis the NS3-PRO mutant showed some protein present in the nuclear fraction. Again, this might be explained by the fact that the ER is continuous with the nuclear membrane. Upon fractionation it is then possible that ER is also present in the nuclear fraction. The membrane flotation analysis confirmed that all of the NS3-PRO protein is membrane associated.

The NS3-CR protein showed a more uniform distribution around and inside the nuclear membrane as well as the formation of intranuclear bodies. With 3-D imaging software of the confocal images it was clear that the intranuclear bodies were associated with the nuclear membrane. The results obtained with the confocal microscope were supported by the findings with the subcellular fractionation and membrane flotation assays for the NS3-CR mutant. It is clear that the conserved region (NS3-CR) also plays an important role in NS3 trafficking. What remains undetermined is whether the conserved region is also involved in any interaction with host cell proteins or whether this region only allows proper folding of the NS3 N-terminal domain enable the binding of the closely located L-domain with host cell proteins.

Some of the results found in my study contradict that of Van Niekerk *et al.* (2001b). It was found from the confocal analysis that NS3A, NS3-PRO and NS3-CR mutants predominantly do not localise to the plasma membrane indicating that these regions do play a role in NS3 trafficking. Cytopathic effects were still observed in infected cells from 48 hpi. One possibility is that these mutants still interact with other intracellular membranes and therefore could affect cell viability. The compromised trafficking of NS3 due to the mutations is also the probable cause of the intranuclear bodies found in cells infected with NS3A and NS3-CR. Van de Merwe (2010) found that in AHSV-2 Bac-NS3 infected cells Sf9 cells, there is a positive correlation between the presence of

intranuclear bodies and cell death. These INBs could be a result of the blocked transport to the plasma membrane. Upon initialization of protein trafficking proteins are sorted in the ER. Here, proteins react with signaling molecules via binding domains to establish trafficking to their target compartments. It is possible that due to the mutations in the signaling regions of NS3, protein sorting signals which are native to baculovirus redirect protein trafficking to the inner nuclear membrane (Braunagel *et al.*, 2009) resulting in the presence of INBs. Another factor that should be kept in mind is that proteins normally found in trace amounts in host cells are overexpressed in the baculovirus expression system. This could also be a possible reason for the differences in trafficking that is observed.

NS3 has two hydrophobic membrane spanning domains which insert into the lipid bilayer so that both the N- and C-terminals project into the cell cytosol (Van Staden *et al.*, 1995). As previously mentioned Van Niekerk *et al.* (2001) found that mutations in either of the two hydrophobic domains hindered the ability of these proteins to insert correctly into the membranes and thereby abolished protein trafficking to the plasma membrane. The following is a summary of what was found for these proteins in my study: The hydrophobic domain mutants were very difficult to detect (Western blot) due to very low expression levels. The NS3-HD1 and NS3-HD2 proteins showed much variability in their localisation patterns when analysed by confocal microscopy. The NS3-HD1 has a punctate cytoplasmic localisation and occasionally a regular smooth plasma membrane distribution. This mutant was never observed in the nuclear membrane. The NS3-HD2 appeared to have a more uniform localisation in the cell cytoplasm and on occasion throughout the cell nucleus. On the rare occasion when NS3-HD2 was observed in the plasma membrane, it had an uneven membrane distribution similar to the WT NS3. Both the eGFP-tagged hydrophobic domain mutants had a uniform unlocalised distribution throughout the cell cytoplasm and nucleus, identical to the WT eGFP protein. With the exception of NS3-HD2, none of the hydrophobic mutants could be detected in any of the membrane assays. Taking all of these results into account no definite conclusions could be drawn. It was evident however that mutations in either of the hydrophobic domains compromised the protein stability, which could result loss of membrane association or protein misfolding and in the case of the eGFP mutants, resulted in protein cleavage. Western blot analysis confirmed that protein cleavage had occurred and that the uniform green localisation that was observed in infected Sf9 cells were only the eGFP proteins and not due to the HD fusion proteins.

Two alternative deletion mutant proteins that were considered in order to determine the roles of specific conserved domains in NS3 were N11-eGFP and NS3 Δ C-eGFP. The N11-eGFP protein consists of the first eleven amino acids of NS3, fused to eGFP on the C-terminal domain whilst the NS3 Δ C-eGFP has a 14 aa C-terminal deletion, fused to eGFP (C-terminus). With confocal, fractionation and membrane flotation analysis the N11-eGFP mutant results were all identical to

WT eGFP however the N11-eGFP was expressed at much higher levels. The N11-eGFP was constructed in order to identify a possible role for the NS3 N-terminal and to try and determine if NS3A makes use of different trafficking mechanisms or pathways due to the lack of the N-terminal. Unfortunately the N11-eGFP mutant could not help resolve any of these questions, probably due to the inability of the 11 aa peptide to counter the soluble effects of the eGFP tag. The truncated NS3 Δ C-eGFP protein showed identical localisation, fractionation and membrane flotation results to the WT NS3 and NS3-eGFP protein indicating that the dileucine motif identified on the C-terminal domain of NS3 does not play any role in trafficking toward the plasma membrane. This could be expected if the trafficking of AHSV NS3 is similar to that of BTV. The C-terminus of BTV NS3 has been shown to interact with newly formed virus particles for trafficking to the plasma membrane for viral release (Beaton *et al.*, 2002).

WT NS3 protein has a punctate perinuclear localisation in the cytoplasm as well as an irregular distribution through the plasma membrane. The perinuclear localisation is putatively ER and Golgi associated. One hypothesis is that since NS3 is a transmembrane protein, it utilizes the host cell secretory pathway for membrane bound proteins for viral egress. In this pathway the membrane protein is inserted into the lipid bilayer of a membranous organelle (ER) and buds from one organelle to the next (Golgi) until it reaches its target destination (plasma membrane) (Lodish *et al.*, 2003). To investigate the trafficking of NS3 in insect and mammalian cells, immunolabelling and colocalisation was done with NS3 and organelle specific antibodies.

Unfortunately we were not able to establish ER labelling despite numerous attempts. Two different ER markers (ER specific anti-calnexin antibody and live cell marker) were used and tested in both Sf9 insect cells as well as Vero, BSR and BHK cells. After a number of repeats it was observed that the anti-calnexin did not bind specifically to the ER whilst the BacMam ER-RFP was observed in live cells, however upon fixation and immunolabelling (according to the manufacturers' protocol) of the NS3 proteins, the ER-RFP signal was lost completely. In order to address this problem, live cell imaging using the BacMam ER-RFP together with a transiently expressed NS3-eGFP in mammalian cells would have to be considered. Alternative cell lines would also be recommended. Similar colocalisation studies in rotavirus have revealed valuable information regarding the viral life cycle (Cheung *et al.*, 2010), viral trafficking and release mechanisms (Hyser *et al.*, 2010; Storey *et al.*, 2007) and cell type dependent mechanisms of NSP4 (Gibbons *et al.*, 2011). Even though these cellular markers proved unsuccessful in this study, the colocalisation of NS3 and other AHSV proteins with cell structures remain one of our future goals.

In order to determine if NS3 proteins interact with the cell cytoskeleton, BSR cell were infected and dual-labelled with anti-NS3 and either a marker for microtubules or vimentin. From the results it was confirmed that NS3 does not interact with microtubules, as expected. This could also be

confirmed by chemical inhibition with colchicines which disrupts the microtubule network. The vimentin labelling had an inhibiting effect on the NS3 labelling. The NS3 antibodies only bound in cells that lacked vimentin labelling. This was unfortunate since we expected partial colocalisation of NS3 with vimentin. In BTV the C-terminal domain of NS3 interacts with VP2 to carry the newly formed virions to the plasma membrane for viral release (Beaton *et al* 2002). VP2 interaction with vimentin has been shown as critical for viral release (Bhattacharya, 2007). Colocalisation of transiently expressed NS3 and VP2 proteins could perhaps resolve this question however previous yeast-two-hybrid assays failed to find any interaction between AHSV NS3 and VP2 (Beyleveld, 2007).

In cells which were dual-labeled with the anti-Golgin97 (Golgi) and NS3, colocalisation of NS3 and the Golgi marker was observed. Variable percentages of colocalisation were calculated using ImageJ software. This partial colocalisation that was observed was expected since NS3 migrates through the Golgi to reach the plasma membrane. On occasion, AHSV infected Vero cells were also treated with BFA which blocks ER to Golgi trafficking. When these cells were immunolabeled with NS3 and Golgi specific antibodies, all the NS3 was found to localise in the cell nucleus while no Golgi labeling was observed. The disruption of the normal NS3 localisation confirms that NS3 uses the cell secretory pathway for membrane proteins for trafficking to the plasma membrane.

In Bac-NS3 infected Sf9 cells that were treated with BFA, the NS3 localisation pattern remained in the punctate perinuclear cytoplasmic structures (putatively ER) whilst no plasma membrane or nuclear membrane localisation was observed similar to the findings of Liu *et al* (2006). In BFA treated, NS3-eGFP expressing Sf9 cells, the fusion protein showed a much more smooth distribution in the cytoplasm and similarly to the treated WT NS3 had no distribution in the nuclear or plasma membranes. In AHSV infected Vero cells, BFA treatment also abolished NS3 trafficking toward the plasma membrane. In some of the Vero cells the NS3 also showed a smoother localisation pattern throughout the cytoplasm and in some cells intranuclear bodies were present. These INBs did not appear to be membrane associated however this remains unconfirmed. These results confirm that NS3 establishes trafficking to the plasma membrane via the ER through the Golgi apparatus.

Chapter 3:

Concluding remarks

CONCLUDING REMARKS

The aim of this study was to study the intracellular localisation and trafficking of AHSV NS3 proteins and to relate this to its role in cytotoxicity and pathogenesis. In order to do this 14 previously constructed mutant NS3 proteins were compared by means of confocal microscopy and membrane association assays. Six of these mutants were non-eGFP NS3 proteins containing mutations in highly conserved regions whilst the other eight contained an eGFP tag on either the N- or C-terminal domains of NS3. The eGFP tagged proteins were compared to non-eGFP counterparts to determine if eGFP can be used as a reporter molecule to study protein trafficking and localisation. Like BTV, it is hypothesized that AHSV NS3 utilizes host cell trafficking mechanisms to carry newly formed virus particles to the plasma membrane where these are released either through viral budding or lytic mechanisms. In order to answer some question regarding the interaction of AHSV NS3 with host cell proteins, colocalisation analysis was done with NS3 proteins and specific cell organelles and these associations were confirmed with pathway inhibitors.

Before any of the analysis could be done, a number of protocols regarding fixation and immunolabelling had to be optimized. The two methods that were tested are both commonly used as protocols in studies for confocal microscopy. From the optimization it was found that both methods have got their advantages and disadvantages and that the choice of fixative depends mainly on the type of cells, whether immunolabelling or reporter molecules are used as well as the properties of the proteins that are being studied.

Protein conservation normally suggests a functional role. By comparing six non-eGFP NS3 mutants, it was found that mutations in any of the conserved regions lead to alteration in the localisation pattern of NS3 only confirming that these regions play an important role in protein function either through correctly folding of the protein and insertion into membranes or by specific interactions with host cell trafficking proteins. Pull-down or yeast-to-hybrid assays would be necessary in order to determine the specific nature of the protein interaction of these conserved regions (NS3-CR and NS3-PRO) however due to the vast amount of host cell proteins that play a role in trafficking of membrane proteins this could be an almost impossible task. In this study it was also confirmed that mutations in either of the hydrophobic domains of NS3 possibly leads to loss of membrane association and therefore fails to migrate to the plasma membrane.

For many studies the eGFP protein has been successfully applied to study the intracellular localisation and trafficking. By comparing eGFP tagged and untagged NS3 proteins it was found that some mutant proteins showed similar localisation profiles whilst other mutants had completely different profiles and in the case of the hydrophobic mutants resulted in protein cleavage. The addition of the eGFP tag to an insoluble protein (membrane proteins) could also increase the

solubility (Yang *et al.*, 1996). This was confirmed by the membrane assays. The results therefor did not give any definitive answer however lead to the conclusion that for some proteins the eGFP tag can provide a useful tool in studying trafficking and localisation but this is dependent on the size of the protein and the mutations that are induced in the functional regions of the protein.

To establish with which organelles the NS3 proteins associate to promote trafficking, colocalisation analysis was performed either through immunolabeling of NS3 and cellular organelles or live cell markers. One of the major problems encountered with the staining of the organelles is that the antibodies seem to be host cell specific which prevented replication of the experiment in both insect and mammalian cells and even between different mammalian cell lines. We were however able to confirm that NS3 colocalises with the Golgi apparatus and by means of pathway inhibition of the ER-Golgi transport, no NS3 was observed in the perinuclear cytoplasm (in Vero cells). Pathway inhibition of ER to Golgi transport in insect cells also prevented NS3 trafficking to the plasma membrane. These findings confirm that NS3 uses the cell secretory pathway for membrane proteins for trafficking to the plasma membrane in both insect and mammalian cells. It is unclear though whether there exist any alternative transport mechanisms for AHSV NS3.

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