Interaction of the African horsesickness virus NS3 protein with selected cellular proteins and its importance in viral egress from mammalian cells

by

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DECLARATION

I declare that the dissertation, which I hereby submit for the degree M.Sc (Microbiology) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signed: …………………………………..   Date: …………………………..

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SUMMARY

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African horsesickness virus (AHSV) is a member of the *Orbivirus* genus within the *Reoviridae* family and causes an acute disease in horses. AHSV encodes four non-structural proteins (NS1, NS2, NS3/NS3A), whose functions in the viral life cycle are not fully understood. Amongst these, NS3 is believed to mediate virus release from infected cells. Data for bluetongue virus (BTV), the prototype orbivirus, has indicated that NS3 recruits cellular proteins to aid in the non-lytic release of virus particles. By making use of yeast two-hybrid screens, it was shown previously that the AHSV NS3 protein interacts with three insect cell proteins, namely Smad Anchor for Receptor Activation (SARA) protein, heat shock protein 70 (Hsp70) and ubiquitin (UB). Based on the involvement of host cell proteins in NS3-mediated non-lytic virus release, the aims of this study were thus to confirm the interactions between AHSV NS3 and the insect cell proteins *in vitro* and to determine whether these proteins may aid virus release from infected mammalian cells.

To confirm the interaction of the AHSV NS3 protein with the insect cell proteins, the SARA, Hsp70 and UB peptides were expressed as glutathione S-transferase (GST)-tagged fusion proteins in *E. coli*. These GST fusion proteins were subsequently used in pull-down assays with *E. coli* lysates containing a truncated AHSV NS3 protein. No interaction between the virus and insect cell proteins could be demonstrated with this assay. To determine whether the SARA, Hsp70 and UB proteins may have biological relevance in the non-lytic release of AHSV from infected cells, a RNA interference (RNAi)-based approach was used. Pre-designed small interfering RNAs (siRNAs) that have been validated for silencing SARA, Hsp70 and UB gene expression in mice were evaluated for their ability to knockdown expression of their target genes in BHK-21 cells. The results indicated that mSARA-siRNA and mUB-siRNA down-regulated transcription of the SARA and UB genes by 63% and 56%, respectively, whereas the mHsp70-siRNA suppressed Hsp70 gene expression by only 3%. BHK-21 cells were subsequently transfected with the respective siRNAs in separate experiments followed by virus infection. Exposure of BHK-21 cells to these siRNAs did not result in a reduction in extracellular virus titres when compared to control cells. Although it is tempting to conclude that the SARA and UB proteins are not functionally relevant with regards to a role in AHSV egress, it is, however, also plausible that these results were due to the inability to reduce the level of target mRNA transcripts to such an extent that it would result in a loss of gene function.

During the course of this study, various parameters that may have potentially influenced the results were identified. Further experiments are thus required before definitive conclusions can be drawn as to whether or not AHSV NS3 interacts with the respective insect cell proteins and, if so, what the relevance of these interactions might be with regards to virus release.

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LIST OF ABBREVIATIONS

AHS	African horsesickness
AHSV	African horsesickness virus
ATP	adenosine triphosphate
BHK-21	Baby hamster kidney-21
BLAST	Basic local alignment search tool
bp	base pair
BSA	bovine serum albumin
BTV	bluetongue virus
°C	degrees Celsius
C	carboxyl terminus
cDNA	complementary DNA
cm	centimetre
C_p	crossing point
CPE	cytopathic effect
Da	Dalton
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleoside-5'-triphosphate
dsDNA	double-stranded DNA
dsRNA	double-stranded RNA
DTT	Dithiothreitol
EBSS	Earle's balanced salt solution
EDTA	Ethylenediaminetetraacetic acid
EEA1	early endosome antigen 1
eIF	eukaryotic translation initiation factor
ER	endoplasmic reticulum
ESCRT	endosomal sorting complex required for transport
FBS	foetal bovine serum
Fig.	figure
FRET	fluorescence resonance energy transfer
FYVE	Fab1p, YOTB, Vac1p and EEA1
GST	glutathione S-transferase
h	hour(s)
H_2O_2	hydrogen peroxide

Hrs	hepatocyte growth factor-regulated tyrosine kinase substrate
Hsc70	heat shock cognate protein 70
Hsp70	heat shock protein 70
Hsp90	heat shock protein 90
IFN	interferon
IPTG	isopropyl β-D-1-thiogalactopyranoside
kb	kilobase pair
kDa	kilodalton
LB broth	Luria-Bertani broth
L-domain	Late-assembly domain
Lys	lysine
MEM	Eagle's Minimum Essential Medium
mg	milligram
miRNA	microRNA
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
MVB	multivesicular body
N	amino terminus
NaOAc	sodium acetate
NEAA	non-essential amino acids
ng	nanogram
nm	nanometre
NP-40	Nonidet-P40
nt	nucleotide
NTPase	nucleoside triphosphate phosphohydrolase
OD_{600}	optical density at a wavelength of 600 nm
OIE	Office International des Épizooties
ORF	open reading frame
p	probability
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction

pfu	plaque forming unit
PI(4,5)P$_2$	phosphatidylinositol (4,5) bisphosphate
PKR	protein kinase R
pmol	picomole
PSB	protein solvent buffer
PtdIns3P	phosphatidylinositol 3-phosphate
qPCR	quantitative polymerase chain reaction
REST	Relative Expression Software Tool
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
RNase	ribonuclease
RNF11	RNF11 RING-H2 E3 ligase
rpm	revolutions per minute
RT-qPCR	real-time quantitative PCR
s	second
SARA	Smad Anchor for Receptor Activation
SD	standard deviation
SDS	sodium dodecyl sulfate
Seg	segment
Sf	*Spodoptera frugiperda*
shRNA	short hairpin RNA
siRNA	small interfering RNA
SNARE	soluble *N*-ethylmaleimide-sensitive fusion attachment protein receptor
SPR	surface plasmon resonance
ssRNA	single-stranded RNA
STAM2	signalling transducing adapter molecule 2
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
TGF-β	transforming growth factor-β
T$_m$	melting temperature
Tris	Tris-hydroxymethyl-aminomethane
Tsg 101	tumour susceptibility gene 101
U	unit
UB	ubiquitin
UV	ultraviolet
V	Volt
v.	version

v/v	volume per volume
VIBs	virus inclusion bodies
VLPs	virus-like particles
w/v	weight per volume
X-gal	5-bromo-4-chloro-indolyl-β-D-galactopyranoside
β2M	β2-microglobulin
μg	microgram
μl	microlitre
μM	micromolar
μm	micrometre

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CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

African horsesickness (AHS), of which African horsesickness virus (AHSV) is the aetiological agent, is a non-contagious, infectious disease that most commonly affects horses. The disease can also occur in other equids such as donkeys and zebra, but they rarely exhibit clinical symptoms (Coetzer and Erasmus 1994; Mellor and Hamblin 2004). AHSV is transmitted to susceptible animals by biting midges of the genus *Culicoides*, which become infected by feeding on animals during the febrile and viraemic stages of infection (Du Toit 1944; Boorman *et al*. 1975; Wilson *et al*. 2009). AHS can manifest different symptoms, including fever, lack of appetite, and lesions that are associated with impairment of the circulatory and respiratory systems (Coetzer and Erasmus 1994). The mortality rate of AHS is dependent on which form of the disease occurs, but has been known to be as high as 95% in naïve horse populations (Coetzer and Erasmus 1994). Due to its potential for high mortality rates in naïve horse populations and its economic impact on the trade and movement of animals between AHS-affected and AHS-free areas, AHS has been listed as a notifiable equine disease by the World organization for animal health (OIE) (MacLachlan and Guthrie 2010; World Organization for Animal Health 2012).

AHS occurs mostly in sub-Saharan Africa, but sporadic incursions into North Africa, the Middle East and India have been reported (Howell 1960; Rafyi 1961; Howell 1963; Gohre *et al*. 1965; Mirchamsy and Hazrati 1973). In recent years, there has been mounting concerns that AHS might spread into Europe, not unlike bluetongue disease (Saegerman *et al*. 2008; Wilson and Mellor 2009), a related disease of ruminants (Spreull 1905; Bekker *et al*. 1934). The movement of AHS into these areas could have a significant economic effect, as the naïve equine populations in Europe would most likely suffer a high rate of mortality (Mellor and Hamblin 2004).

African horsesickness virus (AHSV) is a member of the genus *Orbivirus* in the family *Reoviridae* (Calisher and Mertens 1998), and nine serotypes have thus far been identified (Howell 1962). AHSV has been characterized to a limited extent (Burrage and Laegreid 1994; Skowronek *et al*. 1995) and little is known regarding the molecular basis for the pathogenesis and virulence of the virus. These factors, however, are likely to be complex, as all the steps of viral replication are potentially involved (Laegreid *et al*. 1993; Huismans *et al*. 2004).

The non-structural protein NS3 appears to be an important virulence determinant of AHSV (Van Niekerk *et al.* 2001b; Meiring *et al.* 2009). The NS3 protein of BTV, the prototype orbivirus, has been shown to be essential for the release of BTV particles from infected cells (Hyatt *et al.* 1991; Roy 1992; Hyatt *et al.* 1993). The AHSV NS3 protein has been shown to be targeted to the plasma membrane of mammalian and insect cells (Van Staden *et al.* 1995; Stoltz *et al.* 1996), and it is therefore likely that it may have a similar function to that of the BTV NS3 protein. Furthermore, the carboxyl (C)-terminus of the BTV NS3 protein has been shown to interact with the virus outer capsid protein VP2 (Beaton *et al.* 2002), while the amino (N)-terminus of NS3 has been shown to interact with the cellular proteins p11 (Beaton *et al.* 2002) and tumour susceptibility gene 101 (Tsg101) protein (Wirblich *et al.* 2006). This suggests that the NS3 protein may be involved in the trafficking of virus particles to the cell membrane through its interaction with the virus particle at one terminus and the interaction with membrane-localizing cellular proteins at the other terminus. Although interaction between the NS3 and VP2 proteins of AHSV has not yet been confirmed, the N-terminus has nevertheless been shown to interact with Tsg101. Subsequent silencing of Tsg101 expression was shown to reduce the amount of AHSV that was released from the cells through budding (Wirblich *et al.* 2006).

Towards identifying additional cellular proteins with which the AHSV NS3 protein might interact, a cDNA library was constructed of *Culicoides variipennis* (= *sonorensis*) cells and screened for protein interactions using a yeast two-hybrid system (Barnes 2011). This study indicated that the N-terminus of AHSV NS3 interacts with Smad Anchor for Receptor Activation (SARA), a membrane-targeted cellular protein. A similar study, performed with a *Drosophila melanogaster* cDNA library, indicated interactions with two other cellular proteins, namely ubiquitin (UB) and heat shock protein 70 (Hsp70) (Beyleveld 2007). It therefore follows that further investigations regarding the interaction of the AHSV NS3 protein with these cellular proteins may reveal novel insights into the release of AHSV from infected host cells.

1.2   PATHOGENESIS OF AFRICAN HORSESICKNESS

African horsesickness can manifest as either sub-acute, acute or peracute, depending on the form of the disease (Coetzer and Erasmus 1994; Mellor and Hamblin 2004). Moreover, depending on the virulence of the virus strain and the immunological status of susceptible

animals, the incubation period and severity of the disease can vary. The initial replication of the virus occurs in the lymph nodes, followed by infection of the lungs and lymphoid tissues. Virus replication at these secondary sites gives rise to secondary viraemia that lasts for four to eight days in horses. In donkeys and zebra the period of viraemia may last for up to four and seven weeks, respectively (Coetzer and Erasmus 1994). AHS occurs in four forms, listed here in order of increasing severity: the African horsesickness fever form, the cardiac form, the mixed form and the pulmonary form (Theiler 1921).

African horsesickness fever frequently occurs in horses with some degree of existing immunity or following infection with less virulent AHSV strains, and presents with a moderate fever and oedema of the supraorbital fossae. The animals make a complete recovery and there is no mortality associated with this form of the disease (Mellor and Hamblin 2004). The fever form of AHS is the only form of the disease that has been reported to occur in African donkeys and zebra (Howell 1963). The cardiac or "dikkop" form presents with swelling of the neck and head, especially the supraorbital fossae, but congested conjunctivae, petechial haemorrhages in the eyes and ecchymotic haemorrhages of the tongue may also occur. The mortality rate for the cardiac form is approximately 50% (Coetzer and Erasmus 1994). The mixed form of AHS, which is associated with a mortality rate of approximately 70%, is the most common form of the disease. Affected animals show signs of respiratory distress and oedema typical of the cardiac form of AHS (Coetzer and Erasmus 1994). The pulmonary or "dunkop" form of AHS has the highest mortality rate (approximately 95%), as it most commonly occurs in horses with no pre-existing immunity. Shortness of breath, fits of coughing and the discharge of frothy serofibrinous fluid from the nares typically occur in this form of the disease (Coetzer and Erasmus 1994).

Although there is currently no specific treatment for animals suffering from AHS, the virus is non-contagious and can only be spread via the bites of infected vector species of *Culicoides*. Consequently, the control of AHS may be effected by the introduction of animal movement restrictions to prevent infected animals from initiating new foci of infection and viraemic animals may be slaughtered very early in an epidemic to minimize them acting as a source of virus for vector insects (Mellor and Hamblin 2004). Other approaches aimed at preventing and controlling AHS include vector control (Braverman and Chizov-Ginzburg 1997; Carpenter *et al.* 2008) and vaccination. The currently available vaccine is a polyvalent, live attenuated vaccine produced by Onderstepoort Biological Products, South Africa, but is not

approved for use in Europe due to variable attenuation and weak immunogenicity of some vaccine strains (Mellor and Hamblin 2004).

1.3   EPIDEMIOLOGY AND TRANSMISSION OF AFRICAN HORSESICKNESS

AHS has long been known to be endemic to sub-Saharan Africa, and more specifically, the tropical and sub-tropical regions of sub-Saharan Africa, stretching from Somalia and Ethiopia in the east to Senegal in the west and as far south as the most northern reaches of South Africa (Howell 1963; Binepal *et al*. 1992; Fasina *et al*. 2008; MacLachlan and Guthrie 2010). In South Africa, there is a yearly occurrence of AHS in the north-eastern Lowveld of the Mpumalanga Province, with occasional spread southwards into usually unaffected areas, such Free State and the Cape provinces. The southerly spread of AHS has become less frequent, most likely due to intensive vaccination (Coetzer and Erasmus 1994) and a reduction in the number of zebra, which is considered to be a reservoir host in these regions (Barnard 1998).

AHSV has also been reported to spread into North Africa, the Mediterranean basin and Asia, crossing the significant barrier posed by the Sahara desert (Salama *et al*. 1981; Mellor 1993; Coetzer and Erasmus 1994; Baylis *et al*. 1997). Outbreaks of AHS have also been reported in Europe. During 1965, an outbreak of AHSV-9 first occurred in Morocco before spreading to Algeria and Tunisia and subsequently spread to Spain in 1966 (Diaz Montilla and Panos Marti 1967; Sellers *et al*. 1977). In 1987, an outbreak of AHSV-4, a serotype that had not previously been seen outside southern Africa, was reported in central Spain. This outbreak is believed to have been caused by the importation of sub-clinically infected zebra from Namibia to a safari park near Madrid (Lubroth 1988; Rodriguez *et al.* 1992). The epidemic continued for four months and ended with the onset of colder climatic conditions, suggesting that the virus was incapable of overwintering in Europe. Nevertheless, throughout the years that followed more severe outbreaks of AHSV-4 occurred in Spain (1988, 1989 and 1990), Portugal (1989) and Morocco (1989, 1990 and 1991) (Mellor *et al*. 1990; Mellor 1993; Rawlings *et al*. 1997). Not only have these outbreaks dispelled the long-held belief that AHSV could not survive European winter conditions, but recent expansions in the distribution of competent *Culicoides* insect vectors due to climate change has raised concerns that AHSV may emerge throughout increasingly extensive portions of Europe (Purse *et al.* 2008; Wilson *et al.* 2009).

The transmission of AHSV occurs when a *Culicoides* biting midge takes a blood meal from a viraemic vertebrate host. The virus then replicates in the vector insect after it has penetrated the gut wall, and eventually spreads to the salivary glands from where it can then be transmitted to another vertebrate hosts during a subsequent blood meal (Wilson *et al*. 2009). Thus, in order for the transmission cycle of AHSV to be maintained, both a vertebrate host and a competent vector insect are required. Zebra, which show little to no clinical symptoms of AHSV infection, have long been considered to be the natural vertebrate host of the virus in Africa (Barnard 1993; Coetzer and Erasmus 1994; Wilson *et al*. 2009). Zebra herds may represent a population of vertebrate hosts from which *Culicoides* can become infected and, in turn, infect domesticated horses. However, the occurrence of AHSV in West Africa, where zebra are absent, suggests that they might not be integral to the persistence of AHSV in Africa (Wilson *et al*. 2009).

Culicoides imicola was the first confirmed field vector of AHSV (Blackburn *et al*. 1985). However, AHSV has also been isolated from mixed pools of *C. pulicaris* and *C. obsoletus* in Spain (Mellor *et al*. 1990), and from *C. bolitinos* in the Free State province in South Africa (Meiswinkel and Paweska 2003). *C. imicola* appears to be the primary vector of AHSV in Africa, accounting for over 90% of *Culicoide*s midges caught in light traps (Wilson *et al*. 2009). In South Africa, it has been suggested that *C. bolitinos* may play a role in virus transmission in cooler areas, where *C. imicola* is less prevalent (Venter and Meiswinkel 1994; Meiswinkel and Paweska 2003). Although it has not yet been proved to be a vector in the field, *C. variipennis* (= *sonorensis*), which is prevalent in North America, has been shown to be capable of AHSV transmission under laboratory conditions (Boorman *et al*. 1975).

1.4   AETIOLOGY OF AFRICAN HORSESICKNESS VIRUS

African horsesickness virus (AHSV) is a member of the genus *Orbivirus* in the family *Reoviridae* (Calisher and Mertens 1998). The prefix "orbi" in "orbiviruses" is derived from the Latin word "orbis", meaning ring or circle, and describes the characteristic capsomers (rings) on the orbivirus core-surface (Borden *et al.* 1971). Orbiviruses can be distinguished from other members of the *Reoviridae* in that they replicate in both insects and vertebrates, show greater sensitivity to lipid solvents and detergents, and virus infectivity is lost in mildly acidic conditions (Spence *et al.* 1984).

The AHSV virion, which is approximately 87 nm in diameter (Manole *et al*. 2012), is a non-enveloped particle and consists of two concentric protein layers that enclose the viral genome (Els and Verwoerd 1969; Oellermann *et al*. 1970). The AHSV genome comprises ten double-stranded RNA (dsRNA) segments (Bremer 1976; Bremer *et al*. 1990), each of which encodes at least one polypeptide (Grubman and Lewis 1992). The outer capsid of the virion is composed of the two major structural proteins VP2 and VP5, whereas the inner capsid or core is composed of the structural proteins VP3 and VP7 (Manole *et al*. 2012). The core encloses the dsRNA genome of the virus, as well as the three minor proteins VP1, VP4 and VP6 (Manole *et al*. 2012). In addition to these structural proteins, at least four non-structural proteins (NS1, NS2 and NS3/3A) have also been identified in infected cells (Mertens *et al*. 1984; Roy 1992).

In contrast to AHSV, bluetongue virus (BTV), the prototype orbivirus, has been studied extensively at the molecular, genetic and structural levels (Roy, 2008a). In the following sections, information on AHSV will therefore be supplemented with that obtained from studies undertaken on BTV.

1.4.1 The African horsesickness virus genome

The AHSV genome comprises ten linear dsRNA segments of varying sizes, which can be grouped into large (Seg-1 to Seg-3), medium (Seg-4 to Seg-6) and small (Seg-7 to Seg-10), according to their electrophoretic mobility through a polyacrylamide gel (Oellerman 1970; Bremer 1976). The size of the ten AHSV genome segments ranges from 3965 base pairs (bp) for the largest segment (Seg-1) to 756 bp for the smallest (Seg-10) (Roy *et al*. 1994). Each genome segment is monocistronic, except for Seg-10 that encodes the two related non-structural proteins NS3 and NS3A (Van Staden *et al*. 1991; Grubman and Lewis 1992) and Seg-9 that encodes structural protein VP6 and non-structural protein NS4 (Dr. V. van Staden, personal communication). The genome segments contain conserved 5' (5'-GUU$^{A/}_U$ A$^{A/}_U$…) and 3' (…AC$^{A/}_U$UAC-3') terminal hexanucleotides, which is a feature typical of viruses in the *Reoviridae* family (Roy *et al.* 1994). In addition, each genome segment has segment-specific terminal inverted repeat sequence motifs, which may be important for initiation of transcription, the sorting and assembly of genome segments during replication or they may serve to protect the RNA against degradation (Roy 1989; Thomas *et al*. 1990; Roy *et al*. 1994).

1.4.2   The African horsesickness virus core

The AHSV core particle can be segregated into two distinct layers. The innermost layer of the core particle comprises 120 copies of the triangular-shaped VP3 protein (as seen in Fig. 1.1). The VP3 proteins are arranged as 60 asymmetric dimers to form a continuous scaffold onto which the VP7 protein is deposited. The VP7 protein therefore forms the outer layer of the core particle. This outer layer is composed of 780 VP7 molecules that are organized into 260 trimers (Manole *et al.* 2012). The VP3 layer encloses the dsRNA genome and the three minor core proteins VP1, VP4 and VP6, each of which likely plays a significant role in the replication of the viral genome.
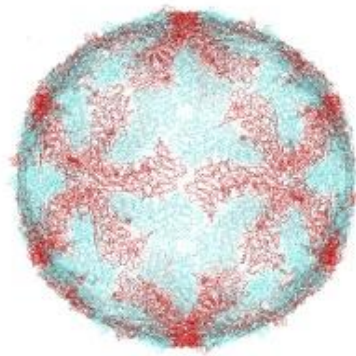
Figure 1.1: The AHSV subcore. The copies of VP3 within an asymmetric dimer are coloured cyan and red, respectively, to illustrate how the dimers are put together to form the subcore (Manole *et al*. 2012).

The VP1, VP4 and VP6 proteins are candidates for the virus-directed RNA polymerase and associated proteins responsible for synthesis of the ten viral mRNAs during the virus infectious cycle. The VP1 protein is likely a RNA-dependent RNA polymerase and harbours a GDD motif typical of RNA polymerases of viruses with a positive sense ssRNA genome and some dsRNA viruses (Roy *et al.* 1988; Vreede and Huismans 1998). The VP1 protein of BTV displays RNA-elongation activity *in vitro* when supplied with a single-stranded poly(U) template and a poly(A) primer (Urakawa *et al.* 1989). The VP1 protein also exhibits replicase activity since it is able to synthesize full-length complementary RNA strands of *in vitro*-synthesized BTV ssRNA templates (Boyce *et al.* 2004; Matsuo and Roy 2013).

During transcription the 5' ends of the viral mRNAs are capped and methylated, thereby resulting in the stabilization and efficient initiation of translation of the viral mRNA (Matsuo and Roy 2013). The VP4 protein of BTV was shown to demonstrate guanylyl transferase (Le Blois *et al.* 1992) and methyltransferase type 1 and type 2 (Ramadevi *et al.* 1998) activities. In addition, VP4 also binds to GTP and displays nucleoside triphosphate phosphohydrolase (NTPase) activity, which is considered to be of importance for transcription and dsRNA processing (Ramadevi and Roy 1998). The AHSV VP4 protein displays sequence identity with the BTV VP4 protein and it has been proposed that it may have similar functions as reported for the BTV VP4 protein (Mizukoshi *et al.* 1993; Roy *et al*. 1994).

The VP6 protein is an integral component of the viral transcription complex and is vital for primary virus replication in infected cells (Matsuo and Roy 2009). The VP6 protein of BTV and AHSV is capable of binding both viral and non-viral ssRNA, as well as dsDNA (Roy *et al.* 1990; De Waal and Huismans 2005). Domains responsible for the nucleic acid-binding activity of the AHSV VP6 protein have been mapped to a region in the centre of the protein and to the C-terminus (De Waal and Huismans 2005). The BTV VP6 protein is an ATP-dependent RNA helicase and is capable of catalyzing the unwinding of dsRNA (Stauber *et al.* 1997). Based on these properties, it is likely that VP6 may be involved in unwinding the dsRNA genome prior to the initiation of transcription and/or may be involved in the encapsidation of the RNA (Stauber *et al.* 1997; Matsuo and Roy 2009).

1.4.3   The African horsesickness virus outer capsid

The outer capsid of AHSV (Fig. 1.2) that surrounds the core of the virion is composed of VP2 and VP5 (Manole *et al*. 2012). Although a reliable homology model of VP5 could not be obtained, Manole *et al.* (2012) suggested that the shape and fold topology of the AHSV VP5 protein may be similar to the VP5 protein of BTV. In the case of BTV, there are 360 molecules of VP5, arranged as 120 globular-shaped trimers, which are positioned on the VP7 trimers (Hewat *et al.* 1992; Stuart and Grimes 2006). There are 180 copies of the AHSV VP2 protein and they form 60 triskelions (a motif consisting of three interlocked spirals) that protrude from the capsid as "sail-shaped spikes" (Manole *et al.* 2012).
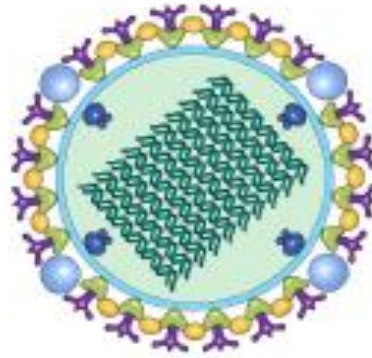
Figure 1.2: The overall structure of AHSV. The genome is surrounded by the subcore, consisting of VP3 (cyan). The transcription complexes, shown in dark blue, are situated just beneath the VP3 layer. The final layer of the viral core consists of VP7, which is shown in green. The outer capsid consists of VP2 (purple) and VP5 (yellow), with the light blue circles representing an unidentified density observed during microscopy (Manole *et al.* 2012).

The VP5 protein of AHSV contains a neutralizing epitope (Martínez-Torrecuadrada *et al.* 1999) and plays a role in enhancing the neutralizing immune response elicited by VP2 in vaccinated horses, possibly through its conformational interaction with VP2 (Martínez-Torrecuadrada *et al.* 1996). Although the exact function of VP5 is not yet known, it has been reported to be cytotoxic when expressed in *Escherichia coli* and *Spodoptera frugiperda* (Sf9) insect cells (Du Plessis and Nel 1997; Martinez-Torrecuadrada *et al.* 1999), and to possess membrane-permeabilizing activity (Hassan *et al.* 2001; Stassen *et al.* 2011). The VP5 protein of BTV has been shown to contain a highly conserved WHXL motif on its C-terminus, which mediates the interaction of VP5 with lipid rafts (Bhattacharya and Roy 2008). These are lipid microdomains within cell membranes that can selectively include or exclude specific proteins (Simons and Ikonen 1997). The WHXL motif is a known soluble *N*-ethylmaleimide-sensitive fusion attachment protein receptor (SNARE) domain (Fukuda *et al.* 2000), which is responsible for mediating association with lipid rafts through interactions with the raft-associated integral plasma protein neurexin (Faivre-Sarrailh *et al.* 2000). BTV VP5 also associates with phosphatidylinositol (4,5) bisphosphate (PI(4,5)P$_2$) (Bhattacharya and Roy 2013), a minor phospholipid that occurs in both the plasma and intracellular membranes, including the Golgi apparatus, endosomes and the endoplasmic reticulum (ER) (Watt *et al.* 2002). When the association of VP5 with PI(4,5)P$_2$ is interrupted, there is a significant reduction in the total viral titre, but there is no apparent effect on either cellular or viral

protein production (Bhattacharya and Roy 2013). It is thus likely that the BTV VP5 protein is involved in the maturation of newly formed virions.

The VP2 protein is the serotype-specific antigen (Huismans and Erasmus 1981) and viral haemagglutinin (Hassan and Roy 1999). It is the least conserved of the structural proteins, most likely due to selective pressures of the host immune system (Potgieter *et al.* 2003). BTV VP2 has been shown to be essential for entry of the virus into host cells (Hassan and Roy 1999). Analysis of the VP2 protein structure has indicated the presence of three putative sialic acid-binding sites located in the "hub domain". Although the direct interaction of BTV VP2 with sialic acid has not yet been demonstrated, a reduction in the amount of sialic acid appears to have a negative impact on viral entry (Zhang *et al*. 2010). It has been suggested that the "tip domain" of VP2 is ideally situated for initial attachment to the host cell membrane, likely by interaction with a glycoprotein, and that binding of sialic acid may stabilize this interaction (Hassan and Roy 1999; Zhang *et al*. 2010). The N-terminus of VP2 has also been shown to interact with vimentin (Bhattacharya *et al*. 2007), an intermediate filament protein that is a major structural component in all metazoan cells (Parry and Steinert 1999) and has been reported to be involved in intracellular trafficking (Ivaska *et al*. 2007). Notably, interruption of the VP2-vimentin interaction resulted in the accumulation of virus particles within the cell, indicating that this interaction is likely involved in the movement of mature virions to the cell surface (Bhattacharya *et al*. 2007). The above results therefore implicate VP2 as playing a role not only in viral entry, but also in viral egress.

1.4.4   African horsesickness virus non-structural proteins

In addition to the structural proteins, AHSV also encodes several non-structural proteins, namely NS1, NS2, NS3/NS3A (Grubman and Lewis 1992) and NS4 (Dr. V. van Staden, personal communication). These non-structural proteins are produced during the virus infectious cycle and are believed to facilitate replication, morphogenesis and exit of progeny virions from infected cells. The following sections provide a brief summary of the NS1, NS2 and NS4 proteins, whereas a more detailed review of NS3 and NS3A is provided in Sections 1.4.5 and 1.6.

During the replication of AHSV, tubules, consisting of multiple copies of NS1, are observed in the infected host cell (Huismans and Els 1979). NS1 expressed by a recombinant

baculovirus in insect cells forms tubules of various lengths (up to 4 µm) with an average diameter of 23 nm. These tubules are reported to have a fine net-like internal structure, while the edges of the tubules are defined and smooth (Maree and Huismans 1997). The function of the NS1 tubules in orbivirus replication is not yet completely understood. It has been proposed that the tubules may be involved in translocation of virus particles from virus inclusion bodies (VIBs) to the cell plasma membrane prior to virus release or that they may prevent the core particle from assembling before the minor structural proteins and/or genome segments have been incorporated (Eaton *et al.* 1990). It has also been suggested that the NS1 protein of BTV could be a major determinant of pathogenesis in the vertebrate host since it augments virus-cell association that leads to lysis of the infected cell (Owens *et al.* 2004). In addition, inhibition of NS1 protein synthesis was reported to cause a switch from lytic virus release to budding of the virions from the plasma membrane. Consequently, it was also proposed that NS1, in conjunction with NS3, may regulate virus release from infected cells (Owens *et al.* 2004). In a recent study, Boyce *et al.* (2012) reported that in BTV-infected mammalian cells the NS1 protein is responsible for mediating the replacement of cellular protein translation with translation of viral genes. Interestingly, these results suggest that NS1 is a positive regulator of viral protein synthesis and this may account for the observed shutdown of cellular gene expression and its simultaneous replacement with viral gene expression in BTV-infected cells.

The NS2 protein is the predominant component of VIBs and expression of NS2, in the absence of other viral proteins in both insect and mammalian cells, results in the formation of inclusion bodies that are indistinguishable from VIBs found in virus-infected cells (Thomas *et al.* 1990; Uitenweerde *et al.* 1995). The VIBs have been shown to contain ssRNA, dsRNA, viral proteins, as well as complete and incomplete virus particles (Eaton *et al.* 1988; Eaton *et al.* 1990). These observations have led to the recognition of VIBs as the sites in which virus assembly occurs, leading to them being termed "virus factories". The NS2 protein has a strong affinity for ssRNA (Huismans *et al.* 1987; Lymperopoulos *et al.* 2003), suggesting that it may have a role in the recruitment and packaging of viral ssRNA prior to encapsidation. NS2 is the only virus-specific protein that is phosphorylated in infected cells (Huismans *et al.* 1987; Devaney *et al.* 1988). In BTV, phosphorylation of NS2 is required for VIB formation and dephosphorylation of the protein is proposed to allow disassembly of the VIBs with subsequent release of the assembled viral cores (Modrof *et al.* 2005). BTV NS2 displays phosphohydrolase (NTPase) activity, and can bind and hydrolyse both ATP and GTP to their

corresponding nucleotide monophosphates (Horscroft and Roy 2000; Taraporewala *et al.* 2001). It has been suggested that the NTPase activity may play a role in providing energy for the assortment, movement, packaging or condensation of bound ssRNA (Horscroft and Roy 2000).

A bioinformatic analysis of various orbiviruses indicated the presence of a second open reading frame (ORF) on genome segment 9, which encodes for the VP6 protein (Firth 2008). The *in vivo* existence of the corresponding protein product, approximately 12 kDa in size, has since been shown for BTV and was designated NS4 (Belhouchet *et al.* 2011). The BTV NS4 protein was shown to be expressed during replication of BTV in both insect and mammalian cells, and to localize to the nucleus and cellular membranes (Belhouchet *et al.* 2011; Ratinier *et al.* 2011). However, the protein does not appear to be essential to the viral replication process. Recently, it was shown that AHSV also encodes a NS4 protein in infected mammalian cells, and synthesis of the NS4 protein was furthermore demonstrated in *Spodoptera frugiperda* (Sf9) insect cells infected with a baculovirus recombinant expressing the Seg-9 open reading frame (Dr. V. van Staden, personal communication).

1.4.5   African horsesickness virus non-structural proteins NS3 and NS3A

The AHSV non-structural proteins NS3 (24 kDa) and NS3A (23 kDa) are both encoded by segment 10, but translation of NS3A is initiated from a second in-frame start codon at nucleotide positions 53 to 55. Thus, NS3A is identical to NS3, excepting that it lacks the N-terminal 11 amino acid residues of NS3 (Van Staden and Huismans 1991). The NS3 proteins are synthesized in lower amounts compared to NS1 and NS2, and they are also synthesized in different amounts depending on the host cells. In contrast to infected insect cells in which NS3 accumulates in high levels, low levels of the protein are expressed in infected mammalian cells (Guirakhoo *et al.* 1995; Van Staden *et al.* 1995).

The NS3 protein of AHSV is not highly conserved between the different AHSV serotypes and represents the second most variable protein encoded by the virus (Bremer *et al.* 1990; Van Niekerk *et al.* 2001a; Van Niekerk *et al.* 2003). Nevertheless, several conserved regions have been identified in the AHSV NS3 protein and are also present in the NS3 protein of BTV (Van Staden *et al.* 1995; Van Staden *et al.* 1998; Van Niekerk *et al.* 2003). These regions comprise an initiation codon for NS3A within the N-terminus, a proline-rich region,

two hydrophobic transmembrane domains (named TM1 and TM2) and a highly conserved amino acid region preceding the first transmembrane domain. These regions and their relative locations are indicated in Fig. 1.3.
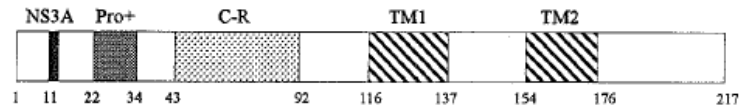
Figure 1.3: Diagram depicting the in-phase initiation codons (position 1 and 11), the proline-rich domain (Pro+), the conserved amino acid region (C-R) and the two hydrophobic regions (TM1 and TM2) of the AHSV NS3 protein. The numbers are indicative of their position on AHSV-3 NS3 (adapted from Van Staden *et al.* 1998).

The hydrophobic regions of the AHSV NS3 protein have been predicted to span the plasma membrane in such a way that both termini are located within the cell cytoplasm (Van Staden *et al*. 1995), as illustrated in Fig. 1.4. This prediction is supported by the inability to detect AHSV NS3 on the surface of intact infected cells (Stoltz *et al*. 1996). NS3 has been shown to have a cytotoxic effect on *S. frugiperda* insect cells (Van Staden *et al*. 1995). This effect is dependent on the two transmembrane domains of NS3, as the cytotoxic effect is abrogated if either of these domains are mutated (Van Niekerk *et al.* 2001b). The cytotoxic effect is hypothesized to be caused by changes in the permeability of the cell membrane, thus resulting in osmotic disregulation and eventual cell death.
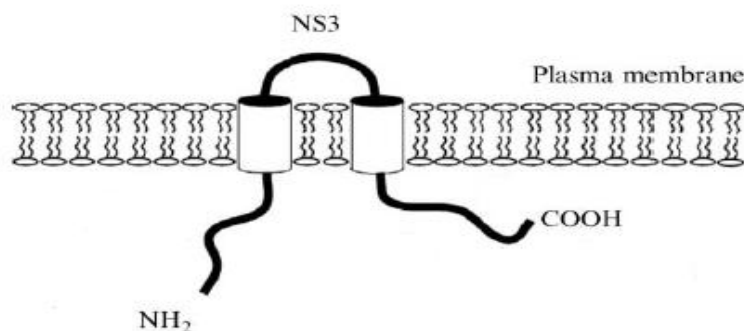
Figure 1.4: Insertion of the hydrophobic domains of BTV NS3 into the cell plasma membrane. Both the N- and C-terminal ends are located within the cytosol of the host cell (adapted from Beaton *et al.* 2002).

In contrast to BTV NS3, which is glycosylated (Wu *et al*. 1992), the AHSV NS3 protein is not, despite the presence of predicted glycosylation motifs in the NS3 proteins of some AHSV serotypes (Van Staden *et al*. 1995; Van Niekerk *et al*. 2001b). Glycosylation of BTV NS3 has been suggested to play a role in stabilizing the protein (Bansal *et al*. 1998). The NS3 protein of both AHSV and BTV has been found associated with the plasma membrane in areas of virus particle extrusion, suggesting that it may be involved in the final stages of orbivirus morphogenesis (Hyatt *et al*. 1991; Stoltz *et al*. 1996). This function has been confirmed in BTV, as the release of virus-like particles (VLPs) occurred only in the presence of cells co-infected with a baculovirus recombinant expressing NS3/NS3A (Hyatt *et al*. 1993). Characterization of AHSV reassortant viruses, in which the S10 genome segment encoding NS3 was exchanged between different AHSV serotypes with or without other genome segments, indicated that exchange of the NS3 gene resulted in changes in virus release, membrane permeability and total virus yield (Meiring *et al.* 2009).

1.5 VIRUS REPLICATION

Since very little is known regarding the infectious cycle of AHSV, the closely related BTV is frequently used as a model for orbivirus replication and morphogenesis (Mertens 2002; Mertens and Diprose 2004; Roy 2008b). Replication of BTV occurs in several stages during which the virus must be taken into the host cell, uncoated to yield a transcriptionally active core particle, replicated and the newly assembled progeny virions must then be released from the infected cell (Fig. 1.5).

During cell entry, BTV VP2 interacts with a receptor on the cell surface, possibly a sialoglycoprotein or sialic acid (Hassan and Roy 1999; Zhang *et al*. 2010), after which the virus is taken up through clathrin-mediated endocytosis and incorporated into early endosomes (Forzan *et al*. 2007). Within the acidic endosomal vesicles, it is possible that the low pH may result in degradation of the VP2 protein, causing the VP5 protein to become completely exposed. The VP5 protein then permeabilizes the endosomal membrane, thereby allowing the transcriptionally active viral core particle to be released into the cytosol (Hassan *et al*. 2001; Forzan *et al*. 2004; Forzan *et al*. 2007).
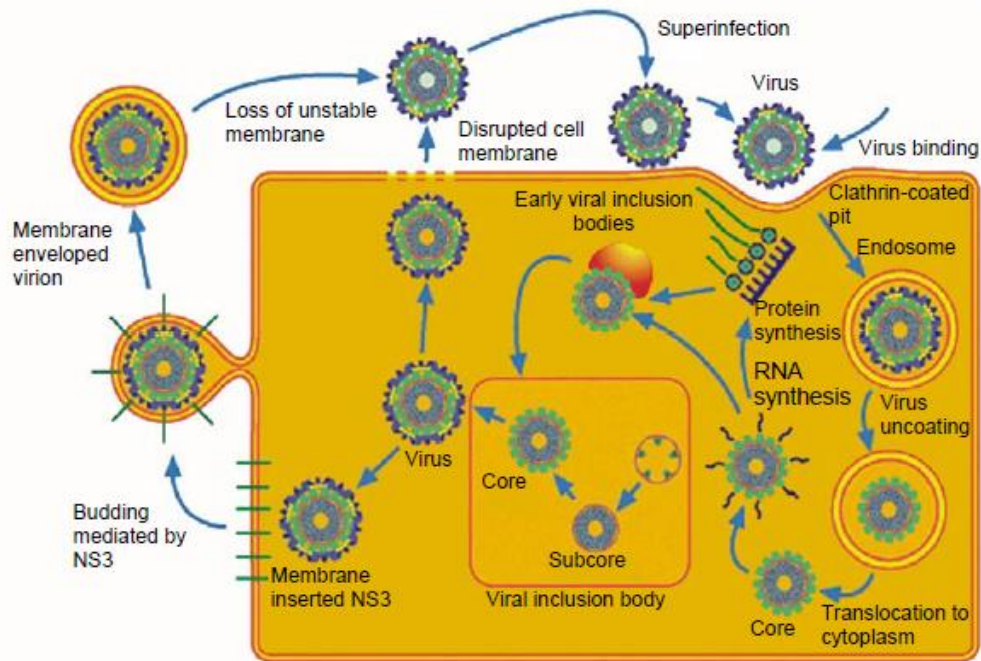
Figure 1.5: Schematic diagram illustrating the infectious cycle of BTV in mammalian cells (Mertens 2002).

The viral genome remains in the core particle where it serves as a template for the continuous production and extrusion of mRNA. This mechanism allows the virus to avoid host interferon defences that are activated by the presence of naked dsRNA in the cytosol (Jacobs and Langland 1996). Following the entry of cellular substrates into the viral core, the viral transcription complex is activated (Mertens and Diprose 2004). As discussed previously (Section 1.4.2), VP6, the viral helicase, likely unwinds dsRNA duplexes ahead of the viral polymerase to enable transcription (Stauber *et al*. 1997). The viral polymerase (VP1) synthesizes ssRNA transcripts of each of the ten RNA genome segments (Urakawa *et al*. 1989; Boyce *et al*. 2004), which are then capped and methylated by the viral capping enzyme VP4 (Ramadevi *et al*. 1998) before being extruded into the cell cytoplasm.

Following translation of viral transcripts, the proteins condense with the viral ssRNA around the parental cores to form granular VIBs. The VIBs can be detected at low levels as early as 10 h post-infection, with a significant increase in the amount of VIBs observed at 12 to 16 h post-infection (Thomas *et al.* 1990). Viral mRNA transcripts, several viral proteins (NS1, NS2, VP1, VP3, VP4, VP6 and VP7), as well as assembled cores and subcores have been identified in the VIBs (Brookes *et al*. 1993; Kar *et al*. 2005; Kar *et al*. 2007). Consequently, it

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is believed that the VIBs represent the sites in which orbivirus replication and early virus assembly occur. Within VIBs, only one copy of each of the ten viral mRNAs (positive strands) is encapsidated within the progeny virions and are subsequently used as templates by VP1 for the production of negative strands to form the dsRNA genome segments (Roy 2008a; Lourenco and Roy 2011; Matsuo and Roy 2013). It is thought that the NS2 protein recruits the viral ssRNA transcripts by interacting with segment-specific hairpin-loop secondary structures (Lymperopoulos *et al*. 2003; Lymperopoulos *et al*. 2006), while VP6 also appears to be essential in the packaging of the ssRNA (Matsuo and Roy 2009).

Maturation of BTV particles is a highly controlled process, involving a specific temporal order of interactions between the structural proteins (Kar *et al.* 2007). Although VP3 is closely associated with the VIBs, the VP7 protein is only recruited to the VIBs in the presence of VP3 where they associate to form stable core particles (Kar *et al.* 2007). The process by which VP2 and VP5 are added to the developing virus particle has not yet been established fully, since these outer capsid proteins do not appear to have an affinity for the VIBs (Modrof *et al.* 2005; Kar *et al.* 2007). It has been suggested that progeny core particles are first produced in the VIBs, then moved to the periphery of the VIBs where they are coated by the outer capsid proteins VP5 and VP2 (Kar *et al.* 2007). However, it was recently reported that VP5 of BTV associates with lipid rafts in the plasma membrane due to the presence of a SNARE domain on the C-terminus of the protein and it was suggested that core particles are transported to these sites for the final assembly of the outer capsid proteins (Bhattacharya and Roy 2008; Bhattacharya and Roy 2013).

Following morphogenesis, the virions are released from infected mammalian cells. Several studies have demonstrated a strong correlation between the presence of NS3 and NS3A, and virus release (Hyatt *et al.* 1993; Stoltz *et al.* 1996). The virions may leave infected cells by budding through the plasma membrane or virions are extruded through a locally disrupted plasma membrane surface (Hyatt *et al.* 1989). The NS3 protein of BTV has also been shown to interact with the cellular proteins p11 and Tsg101, and these interactions were shown to assist egress of virus particles from infected cells in a non-lytic manner (Beaton *et al.* 2002; Wirblich *et al.* 2006; Celma and Roy 2011). The role of NS3 in virus release and the involvement of different cellular proteins in the non-lytic release of virions from infected cells will be discussed in greater detail in the following sections.

1.6   ROLE OF NS3 IN VIRAL EGRESS

As indicated above, the non-structural protein NS3 has been implicated as playing an important role in the late stages of virus morphogenesis by facilitating the release of progeny virions from infected cells either through budding or extrusion through the locally disrupted plasma membrane. It has been proposed that virus release may be a consequence of the inherent properties of the NS3 protein. In this regard, it is interesting to note that BTV NS3 has been suggested to act as a viroporin based on its ability to form homo-oligomers, localization to the plasma membrane and Golgi apparatus of infected cells, and its ability to increase plasma membrane permeability (Han and Harty 2004). Considering that the NS3 protein of both AHSV (Van Staden *et al.* 1995; Stoltz *et al.* 1996) and BTV (Hyatt *et al.* 1991) is localized in plasma membranes, it is therefore conceivable that NS3 may facilitate lytic virus release through destabilization of the plasma membrane.

Although virus release is observed in mammalian cells, the virions remain mainly associated with cellular components and only a minority of virus particles are found in the extracellular medium. This is presumably due to release as a consequence of cytopathic effect (CPE) and lysis of infected cells (Guirakhoo *et al.* 1995). In contrast to mammalian cells, infection of insect cells results in persistent infection with no CPE and the majority of progeny virions are released into the supernatant (Homan and Yunker 1988; Wechsler and McHolland 1988; Guirakhoo *et al.* 1995). Although these results suggest that different strategies may be used to escape from mammalian and insect cells, no studies have as yet been undertaken to unravel the mechanism underlying virus release from insect cells. However, several studies have indicated that the NS3 protein interacts with different cellular proteins that ultimately results in the release of virus particles from infected cells in a non-lytic manner (Beaton *et al.* 2002; Wirblich *et al.* 2006; Celma and Roy 2011).

By making use of a yeast two-hybrid system, Beaton *et al.* (2002) screened a human cDNA library for proteins interacting with BTV NS3 and it was reported that a region spanning the first 13 amino acids of NS3 (and thus absent in NS3A) interacts with protein p11 (calpactin light chain). Calpactin is a tetrameric protein, composed of two light chains (p11) and two heavy chains (Annexin II, also known as p36), which is involved in Ca^{2+}-dependent exocytosis and the trafficking of proteins out of the cell (Gerke 1990; Johnsson *et al.* 1990; Nakata *et al.* 1990; Sarafian *et al.* 1991). Further studies regarding the NS3-p11 interaction

indicated that NS3 interacts with p11 at the same site as p36, thus suggesting that NS3 may recruit p11 in the place of p36 (Beaton *et al.* 2002). This interaction may therefore help to either direct NS3 to sites of cellular exocytosis or it could be part of an active extrusion process. In this regard, release of progeny virions from BTV-infected insect cells was shown to be significantly reduced in instances where the interaction between NS3 and p11 was impaired or abrogated and virus was observed to accumulate in the host cytosol (Beaton *et al*. 2002; Celma and Roy 2011).

In addition to its interaction with protein p11, NS3 has also been shown to be capable of interacting with Tsg101. Using pull-down assays, it was shown that the NS3 and NS3A proteins of BTV, as well as the NS3 protein of AHSV-6 bound to human Tsg101 and also to its orthologue from *Drosophila melanogaster* (Wirblich *et al*. 2006). The Tsg101 protein is a component of the ESCRT-I complex (Katzmann *et al*. 2001), which has been implicated in the intracellular trafficking and non-lytic release of several viruses, including human immunodeficiency virus (HIV), Ebola virus and Marburg virus (Martin-Serrano *et al*. 2001; Urata *et al*. 2007). Investigations regarding the interaction of BTV NS3 with Tsg101 indicated that the interaction is mediated by a PSAP late assembly (L)-domain present on the N-terminus of the NS3 protein, since mutagenesis of this domain abrogated the interaction between BTV NS3 and Tsg101 (Wirblich *et al.* 2006). Notably, a similar L-domain (ASAP) is also present on the AHSV-6 NS3 protein and may thus facilitate interaction with the Tsg101 protein. Furthermore, silencing of Tsg101 gene expression in virus-infected HeLa cells by RNA interference (RNAi) indicated a dramatic reduction in the release of both BTV and AHSV. The release of BTV was reduced by between 65 and 80%, while the release of AHSV was reduced by 50 to 60% (Wirblich *et al*. 2006). The relevance of the NS3-Tsg101 interaction with regards to virus release was also substantiated in studies performed by Celma and Roy (2009). By making use of a mutant virus in which mutations were introduced into the L-domain of BTV NS3 to abrogate NS3-Tsg101 interaction, it was reported that the virion particles were arrested in the process of virus budding as they remained tethered in the cell membranes (Celma and Roy 2009).

1.7   CELLULAR PROTEINS INTERACTING WITH AHSV NS3

The results of the above studies indicated that the orbivirus NS3 protein is capable of interacting with different cellular proteins in order to facilitate virus budding from infected host cells. In an attempt to determine whether AHSV NS3 interacts with other cellular proteins representative of the export machinery in insect cells, yeast two-hybrid screening analyses were performed previously on cDNA libraries from *Culicoides variipennis* (KC) insect vector cells (Barnes 2011) and *Drosophila melanogaster* (Beyleveld 2007). The results of these studies indicated that the AHSV NS3 protein binds to the Smad Anchor for Receptor activation (SARA) protein, ubiquitin (UB) and heat shock protein 70 (Hsp70). Since these cellular proteins are central to this study, their properties will be discussed in greater detail in the following sections.

1.7.1   Smad Anchor for Receptor Activation (SARA) protein

The Smad Anchor for Receptor Activation (SARA) protein, also known as the Zfyve9 protein (Tsukazaki *et al*. 1998; Weiss and Attisano 2013), is a soluble cytoplasmic FYVE domain-containing protein and is found in both insect (Bennet and Alphey 2002; Bökel *et al.* 2006) and mammalian (Tsukazaki *et al.* 1998; Hayes *et al.* 2002 Szymkiewicz *et al.* 2004) cells. The FYVE (Fab1, YOTB, Vac1p and EEA1) domain, of which the name has been derived from the first four proteins identified to harbour this motif (Stenmark *et al.* 1996), is a zinc finger-like motif of 60-80 amino acid residues that binds to the membrane lipid phosphatidylinositol-3-phosphate (PtdIns(3)P) with high specificity (Burd and Emr 1998; Gaullier *et al.* 1998; Patki *et al.* 1998; Blatner *et al.* 2004). PtdIns(3)P is highly enriched in limited (outer) membranes of the early endosomes (Panopoulou *et al.* 2002; Gillooly *et al.* 2003; Bökel *et al.* 2006), and it has thus been suggested that proteins containing FYVE domains contribute to the trafficking of proteins associated with them to these locations (Downes *et al.* 2005; Vicinanaza *et al.* 2009). In addition to facilitating membrane localization of proteins, FYVE domain-containing proteins have also been implicated in regulating the cytoskeleton and signal transduction (Gillooly *et al.* 2001). Indeed, SARA is proposed to mediate transforming growth factor-β (TGF-β) signalling in mammalian cells by recruiting the signal transducer proteins Smad2 and Smad3, thereby enabling these proteins to interact with the TGF-β receptor on the early endosomal membrane and initiate signal transduction (Tsukazaki *et al.* 1998; Itoh *et al.* 2002). TGF-β is a multifunctional cytokine involved during regulation of cell proliferation, differentiation, apoptosis and development

(Roberts and Sporn 1993; Akhurst *et al.* 1998; Massagué 2000). In *Drosophila* cells, SARA has been implicated in decapentaplegic (Dpp) signal transduction, which is an insect homologue of TGF-β (Entchev *et al.* 2000; Bennet and Alphey 2002).

In addition to SARA, two other FYVE domain-containing proteins are also localized to early endosomes, namely the EEA1 (early endosomal antigen 1) and Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) proteins. The SARA protein was reported to co-localize with EEA1, which regulates endosome fusion (Jahn *et al.* 2003) and is an effector of the Rab5 protein (Mills *et al.* 1998; Christoforidis *et al.* 1999). Interaction of the Rab5 and EEA1 proteins has been shown to be involved in the docking and fusion of endosomal vesicles to early endosomes (Simonsen *et al.* 1998; Christoforidis *et al.* 1999). The co-localization of EEA1 with SARA led to the suggestion that SARA may play a general role in membrane trafficking (Hu *et al.* 2002; Bakkebø *et al.* 2012). Considering that no membrane-targeting signals has been reported for orbivirus NS3 proteins, the finding that NS3 binds to SARA (Barnes 2011) may suggest a role for SARA in targeting NS3 to cellular membranes.

The Hrs protein has been shown to bind multiple proteins, including the signal transducing adaptor molecules Smad2 (Miura *et al.* 2000), STAM (Asao *et al.* 1997; Bache *et al.* 2003) and Tsg101 (Kostaras *et al.* 2012). Binding of Smad2 suggests that Hrs co-operates with SARA to stimulate TGF-β expression (Miura *et al.* 2000), whereas the Hrs-STAM complex is known to mediate sorting of proteins for lysosomal degradation (Raiborg and Stenmark 2002; Raiborg *et al.* 2007). In addition, Hrs also facilitates recruitment of the ESCRT protein complexes to the surface of early endosomal membranes via its interaction with the Tsg101 and HCRPI/Vps37 components of the ESCRT-I complex (Katzmann *et al.* 2003; Stuchell *et al.* 2004; Eastman *et al.* 2005; Kostaras *et al.* 2012). This is subsequently followed by recruitment of the ESCRT-II and ESCRT-III complexes in a series of events that ultimately results in the inward budding of the limiting membrane to form the intraluminal vesicles of multivesicular bodies (MVB) (Katzmann *et al.* 2002; Gruenberg and Stenmark 2004; Hurley and Emr 2006). Consequently, Hrs appears to play the role of a scaffold protein that sets the stage for a chain of multiple interactions that takes place at the endosomal membrane (Raiborg and Stenmark 2002; Katzmann *et al.* 2003; Lu *et al.* 2003).

In addition to the above, SARA was recently shown to interact with two proteins, namely signalling transducing adapter molecule 2 (STAM2) and RNF11 RING-H2 E3 ligase

(RHF11) (Kostaras *et al.* 2012). While Tsg101 forms part of the ESCRT-I complex, the STAM2 protein is a core protein of the ESCRT-0 complex, which is responsible for the recruitment of the ESCRT-I complex to sites of MVB formation (Bache *et al.* 2003; Katzmann *et al.* 2003; Lu *et al.* 2003). The RNF11 protein is a small protein containing both a PY motif and a RING-H2 domain, and has been reported to participate in diverse cellular functions, including NF-κB signalling (Shembade *et al.* 2009). The RNF11 protein was also shown to interact with STAM2, as well as with Eps15b, an ESCRT-0 protein (Raiborg and Stenmark 2009). Consequently, it has been suggested that the SARA and RNF11 proteins could therefore also occur in the ESCRT-0 complex.

Interestingly, vesicle budding into the MVB and viral budding at the plasma membrane are considered to be topologically equivalent and it has been proposed that the same cellular machinery could, in principle, catalyze both these processes (Pornillos *et al.* 2002; Calistri *et al.* 2009). Because NS3 of BTV and AHSV carries a late domain motif (PSAP and ASAP, respectively) and binds to mammalian Tsg101, as well as its insect orthologue, it has been proposed that NS3 may recruit the ESCRT machinery to facilitate virion particle budding and release (Wirblich *et al.* 2006). The localization of SARA to Hrs-enriched endosomes in both mammalian (Raiborg *et al.* 2001; Sachse *et al.* 2002) and insect (Bökel *et al.* 2006) cells may therefore be of interest given the reported relationship between Tsg101 and NS3. Moreover, it was recently reported that Hrs, in addition to a PSAP late domain motif, contains multiple independent Tsg101 binding sites (Bouamr *et al.* 2007). It is therefore tempting to speculate that the early endosomes may be enriched with Tsg101 and, following transport of NS3 to these sites via its association with SARA, it may be more readily accessible for interaction with NS3.

It is also important to note that although early work suggested that endocytosis is required for the SARA-TGF-β receptor-Smad complex to form (Tsukazaki *et al.* 1998), several subsequent reports have indicated that this complex can occur independent of receptor internalization (Lu *et al.* 2002; Penheiter *et al.* 2002; Di Guglielmo *et al.* 2003; Bakkebø *et al.* 2012). Consequently, two distinct signaling pathways have emerged in which SARA is proposed to facilitate interaction of Smad2 and Smad3 with the TGF-β receptor at both the early endosome and plasma membrane (Massagué *et al.* 2005; Knoblich *et al.* 2006; Sorkin and von Zastrow 2009). The latter is furthermore supported by immunofluorescent profiles that have demonstrated that SARA and the TGF-β receptors co-localize at the plasma

membrane (Stenmark and Aasland 1999; Penheiter *et al.* 2002). Thus, in an alternative pathway to that above, it could be that NS3 bound to SARA is transported directly to areas in the plasma membrane where the TGF-β receptors reside. Considering the close proximity of early endosomes to the periphery of both *Drosophila* insect cells (Bökel *et al.* 2006) and mammalian cells (Katzmann *et al.* 2002), it may be that NS3 could still be able to recruit Tsg101 and the rest of the ESCRT machinery from these sites.

1.7.2 Ubiquitin

Ubiquitin is a small, highly conserved protein that occurs freely in the cell or is covalently linked to lysine (Lys) residues in substrate proteins (Hershko and Ciechanover 1998; Haglund and Dikic 2005). The attachment of ubiquitin to substrate proteins occurs in three steps. Briefly, a glycine residue on the C-terminus of free ubiquitin is activated by an E1-type enzyme in an ATP-dependent manner and is then followed by the transfer of the ubiquitin molecule to a cysteine residue on ubiquitin carrier protein type E2. Finally, an ubiquitin ligase E3 protein catalyzes the formation of a covalent bond between the ubiquitin carrier protein and a lysine residue in the substrate protein (Hershko *et al*. 1983).

Substrate proteins can be "tagged" with either one or multiple ubiquitin molecules, termed mono- and multiubiquitination, respectively, or they can be "tagged" with chains composed of multiple molecules of ubiquitin, referred to as polyubiquitination (Haglund *et al.* 2003). Polyubiquitin chains are formed when ubiquitin is covalently linked to the lysine residues occurring in ubiquitin itself. While there are seven lysine residues in the ubiquitin protein, the linkages formed on Lys-48 and Lys-63 are the best characterized. Lys-48 polyubiquitination targets proteins for degradation in the 26S proteasome (Chau *et al*. 1989), while Lys-63 polyubiquitination is involved in non-proteasomal functions such as DNA damage control (Spence *et al*. 1995) and protein trafficking (Galan and Haguenauer-Tsapis 1997; Fisk and Yaffe 1999). Monoubiquitination has also been found to be involved in several processes in addition to protein degradation, including endocytosis of plasma membrane proteins and sorting of proteins to MVB (Roth and Davis 1996; Hicke and Riezman 1996; Reggiori and Pelham 2001; Urbanowski and Piper 2001). The different types of ubiquitination, together with their associated functions, are indicated in Fig. 1.6.
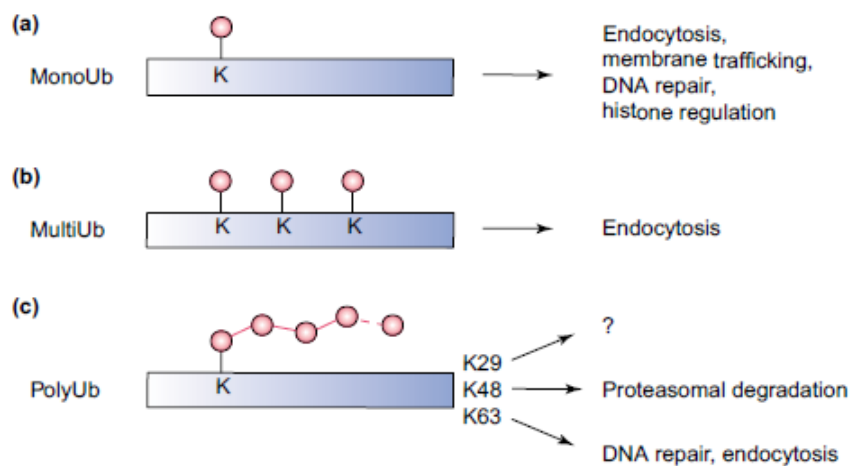
Figure 1.6: An illustration of different types of ubiquitination. (a) Monoubiquitination occurs where a single ubiquitin (pink circle) is covalently bound to a lysine residue (K) on the substrate protein, (b) multiubiquitination is the attachment of single molecules of ubiquitin to more than one lysine residue in the substrate protein, while (c) polyubiquitination is the formation of a chain of multiple ubiquitin molecules attached on a single lysine residue. The functions of the different types of ubiquitination are also indicated (adapted from Haglund *et al.* 2003).

Interestingly, depletion of free ubiquitin in the cell through inhibition of the proteasome has been shown to influence budding of retroviruses (Patnaik *et al*. 2000; Schubert *et al*. 2000; Strack *et al*. 2000; Morita and Sundquist 2004), albeit that the mechanism of action remains unknown. In addition to retroviruses, ubiquitination has also been shown to influence the replication of some negative strand RNA viruses. Inhibition of ubiquitination of the Ebola virus matrix protein VP40 reduces the release of VLPs from cells (Okumura *et al*. 2008), while interfering with the proteasomal pathway has been shown to negatively impact the budding of some paramyxoviruses (Schmitt *et al*. 2005; Watanabe *et al*. 2005). Ubiquitination has been shown to be important in the translocation of the Nipah virus matrix protein from the nucleus (Wang *et al*. 2010), while ubiquitination of the parainfluenza virus 5 matrix protein is thought to be involved in virus assembly, rather than budding (Harrison *et al*. 2012).

1.7.3   Heat shock protein 70 (Hsp70)

Heat shock proteins are a highly conserved group of proteins that are expressed in both eukaryotes and prokaryotes (Lindquist and Craig 1988). These proteins are divided into several families, based on shared molecular weight and sequence homology. Several of the

heat shock protein families have been implicated in the replication cycle of various viruses, with some viruses even encoding their own heat shock protein-like proteins (Agranovsky *et al*. 1991; Hu and Seeger 1996; DeCaprio 1999; Saphire *et al*. 2000).

Although there is currently no published reports regarding the interaction of orbivirus proteins with heat shock proteins, there have been reports on the involvement of heat shock proteins in the replication of rotavirus, another member of the *Reoviridae* family. Three different heat shock proteins, namely heat shock protein 70 (Hsp70), heat shock protein 90 (Hsp90) and heat shock cognate protein 70 (Hsc70), have all been implicated as playing a role in the rotavirus replication cycle (Guerrero *et al*. 2002; Broquet *et al*. 2007; Dutta *et al*. 2009). Both the Hsp90 and Hsc70 proteins were shown to up-regulate viral replication. The Hsc70 protein was reported to be involved in rotavirus cell entry post-attachment (Guerrero *et al*. 2002; López and Arias 2004; Perez-Vargas *et al*. 2006), whereas Hsp90 is thought to be responsible for the downstream activation of cellular factors that regulate cap-dependent translation (Dutta *et al*. 2009).

In contrast to the above, the Hsp70 protein is implicated in the down-regulation of rotavirus replication. This was evidenced by RNAi-mediated silencing of Hsp70 expression in rotavirus-infected Caco-2 cells resulting in a significant increase in the amount of virus structural proteins, as well as a corresponding increase in the production of virions (Broquet *et al*. 2007). The increase in the amount of viral proteins was linked to a reduction in ubiquitination of the rotavirus proteins VP2, VP4 and VP6. Consequently, it was suggested that Hsp70 regulates the levels of viral proteins by targeting the proteins to the sites of ubiquitin-dependent protein degradation. It therefore follows that failure of virus proteins to enter into a degradation pathway would lead to an increase in rotavirus virions (Broquet *et al*. 2007). Despite being a negative regulator of rotavirus replication, it is interesting to note that Hsp70 has been shown to play a beneficial role in the replication of enterovirus (Glotzer *et al*. 2000), and to facilitate capsid assembly of polyoma virus (Chromy *et al*. 2003) and adenovirus (Macejak and Sarnow 1992). Moreover, interaction of Hsp70 with the E1 protein of human papillomavirus was shown to be required for the assembly of pre-initiation complexes at the origin of DNA replication (Liu *et al*. 1998).

1.8   AIMS OF THIS STUDY

Based on the above review of the literature, it is clear that several aspects regarding orbivirus release still need to be elucidated. Recent evidence has implicated the non-structural protein NS3 in virus release (Hyatt *et al.* 1989; Soltz *et al.* 1996; Meiring *et al.* 2009). Moreover, it was shown that NS3 interacts with different mammalian cell proteins such as p11 (Beaton *et al.* 2002; Celma and Roy 2011) and Tsg101 (Wirblich *et al.* 2006; Celma and Roy 2009), as well as with insect cell proteins such as Hsp70, ubiquitin and SARA (Beyleveld 2007; Barnes 2011). In the case of the latter, the biological relevance of these interactions and their contribution to virus release, if any, remains to be determined.

The finding that the AHSV NS3 protein interacts with SARA may have some significance. Notably, SARA has been shown to co-localize with the TGF-β receptors at the plasma membrane and early endosomes (Stenmark and Aasland, 1999; Penheiter *et al.* 2002). Consequently, trafficking of NS3 by SARA to the early endosomes, which are enriched with the Hrs protein (Raiborg *et al.* 2001; Bökel *et al.* 2006) and possesses multiple independent binding sites for Tsg101 (Bouamr *et al.* 2007), may thus provide a means whereby NS3 has an increased likelihood of binding to Tsg101 through its late domain motif. Alternatively, SARA may allow for targeting of the NS3 protein directly to the plasma membrane through the association of SARA with TGF-β receptors located at the plasma membrane.

Ubiquitin plays a role in the targeting of proteins to different pathways, e.g. degradation, protein trafficking and endocytosis pathways (Chau *et al*. 1989; Hicke and Reizman 1996; Fisk and Yaffe 1999; Urbanowski and Piper 2001). In addition, ubiquitin depletion studies have shown that ubiquitin is required for the release of retroviruses from infected host cells (Patnaik *et al.* 2000; Morita and Sundquist 2004; Watanabe *et al.* 2005). Whether the interaction between AHSV NS3 and ubiquitin may play a similar role warrants further investigation.

The Hsp70 proteins are one of the most highly conserved groups of chaperone proteins (Lindquist and Craig 1988). Although Hsp70 has not been implicated in virus release, it is typically involved in the correct folding of proteins, disassembly of protein complexes, intracellular transport and intracellular protein degradation (Bukau and Horwich 1998; Hartl and Hayer-Hartl 2002). Since Hsp70 is involved in targeting proteins either to degradation

pathways or to other pathways for further protein processing (Haglund and Dikic 2005; Hoeller *et al*. 2006), it is possible that the role of Hsp70 during viral replication may be to determine the level of ubiquitination of viral proteins and thus the pathway to which they are targeted. As such, Hsp70 may either up- or down-regulate virus replication.

Towards the long-term goal of mapping the role of NS3 in the AHSV infectious cycle, the specific aims of this study were as follows:

- To confirm interaction of the AHSV-3 NS3 protein with the cellular proteins SARA, ubiquitin and Hsp70 *in vitro* by making use of pull-down assays.

- To determine the effect of silencing expression of SARA, ubiquitin and Hsp70 on AHSV egress from mammalian cells by making use of an RNAi-based approach.

CHAPTER 2

IN VITRO INTERACTION OF THE AHSV NS3 PROTEIN WITH DIFFERENT INSECT CELL PROTEINS

2.1 INTRODUCTION

In contrast to other vertebrate-infecting members of the *Reoviridae* family, members of the *Orbivirus* genus, including African horsesickness virus (AHSV) and Bluetongue virus (BTV), are able to replicate in both the vertebrate host and vector insect (Calisher and Mertens 1998; Wilson *et al.* 2009). The effect of orbivirus replication in these distinct host types is, however, markedly different. For both BTV and AHSV, replication of the virus in insect cells results in persistent infection with no apparent cytopathic effect (CPE), while infection of mammalian cells results in cell death (Guirakhoo *et al.* 1995; Stassen *et al.* 2012). This therefore implies that different mechanisms could be involved in the trafficking of progeny virions out of the infected cells. Indeed, virus particles may either bud through the cell membrane or move through local disruptions of the plasma membrane (Hyatt *et al.* 1989; Stoltz *et al.* 1996). The non-structural protein NS3 of orbiviruses is implicated in aiding virus release from infected cells (Hyatt *et al.* 1989; Hyatt *et al.* 1991; Stoltz *et al.* 1996; Meiring *et al.* 2009). For BTV, data furthermore indicate that NS3 uses host cell proteins such as the p11 subunit of the heterotetrameric calpactin complex (Beaton *et al.* 2002; Celma and Roy 2011) and Tsg101 (Wirblich *et al.* 2006) to facilitate non-lytic release of progeny virions.

In contrast to BTV NS3, limited information is available regarding the interaction of AHSV NS3 with host cell proteins. It has been reported that AHSV-6 NS3 can interact *in vitro* with Tsg101, a cellular component of the ESCRT-I complex (Wirblich *et al.* 2006). More recently, the yeast two-hybrid system (Fields and Song 1989; Chien *et al.* 1991) was used to identify insect cell proteins capable of interacting with AHSV NS3. Using this system, three different proteins from *Drosophila melanogaster* and *Culicoides variipennis* (= *sonorensis*) cDNA libraries were identified that interacted with a truncated AHSV NS3 protein comprising of the N-terminal region only. The interacting cellular proteins were identified as Smad Anchor for Receptor Activation (SARA) protein, heat shock protein 70 (Hsp70) and ubiquitin (UB) (Beyleveld 2007; Barnes 2011). Despite the usefulness of yeast two-hybrid library screens as a means to provide an overview of potential protein-protein interactions, false positive results may be obtained (Serebriiskii *et al.* 2000). Consequently, it is important that interactions that have been identified in yeast two-hybrid screens are confirmed by at least one alternative, non-yeast–based assay. It has been recommended that such methods should preferably consist of an *in vitro* biochemical method or an *in vivo*/*in situ* method (Phizicky and Fields 1995; Shoemaker and Panchenko 2007a; Shoemaker and Panchenko 2007b). Whereas *in vitro*

biochemical methods, such as co-immunoprecipitation or pull-down assays, allow for the study of physical protein-protein interactions (Luban and Goff 1995), the *in vivo/in situ* methods allow insight into possible co-expression and co-localization of the two proteins involved, but generally do not provide conclusive evidence for direct interaction (Lalonde *et al.* 2008; Brückner *et al.* 2009).

Among the various biochemical methods that have been used to validate protein-protein interactions, pull-down assays have been used frequently (Formosa *et al.* 1991; Wirblich *et al.* 2006; Kanno *et al.* 2010; Stack and Bowie 2012; Suzuki *et al.* 2013). This approach relies on immobilizing a protein of interest to a matrix, which is then used to select for ligand proteins from the appropriate cell extract (Schechtman *et al.* 2003). The first step therefore requires the expression of the protein of interest as a fusion protein with a tag moiety. After binding of the recombinant tagged protein to a suitable matrix that serves as a solid phase, the mixture is incubated with a whole-cell lysate or a purified protein and the non-bound material is then washed away. The binding complex is subsequently eluted, the mixture is resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the interacting protein can then be identified by Coomassie staining, Western blotting (in cases where the interacting protein has been previously identified) or mass spectrometry (Phizicky and Fields 1995; Schechtman *et al.* 2003; Ewing *et al.* 2007).

Based on the above, the aim of this part of the study was primarily to confirm the interaction of the AHSV NS3 protein with the insect cell proteins identified previously by means of yeast two-hybrid screens. For this purpose, the interacting SARA, Hsp70 and UB peptides were expressed as glutathione S-transferase (GST)-tagged fusion proteins in *E. coli*, and then used in GST pull-down assays together with a truncated AHSV NS3 protein.

2.2   MATERIALS AND METHODS

2.2.1   Bacterial strains and plasmids

Four different *Escherichia coli* strains were used during the course of this study. *E. coli* JM109 was used as cloning host, whereas *E. coli* BL21(DE3), *E. coli* BL21-Star(DE3) and *E. coli* BL21-Gold(DE3) were used as expression hosts. The expression hosts were a kind gift from Dr. L-M. Birkholtz (Department of Biochemistry, University of Pretoria). All bacterial

cultures were grown in Luria-Bertani (LB) broth, consisting of 1% (w/v) tryptone, 0.5% (w/v) yeast extract and 1% (w/v) NaCl at a pH of 7.4 (Sambrook and Russell 2001). The bacterial cultures were incubated in an orbital shaker at 37°C and 200 rpm. Bacterial cultures were maintained at 4°C on LB agar (LB broth containing 1.2% [w/v] bacteriological agar) or at -70°C as 15% (v/v) glycerol cultures.

Recombinant plasmids containing gene fragments relevant to this study were provided by Dr. V. Van Staden (Department of Genetics, University of Pretoria) and Mr W. Barnes (Department of Microbiology and Plant Pathology, University of Pretoria). These were pGEM-NS3Nterm and pACT-SARA, which contain the N-terminal region (nucleotides 1-355) of the AHSV-3 NS3 gene and a 182-bp fragment of the *Culicoides variipennis* SARA gene, respectively. The recombinant plasmids Clone 2 and Clone 346 were derived from a *Drosophila melanogaster* cDNA library, and contain a 625-bp fragment of the ubiquitin (UB) gene and a 657-bp fragment of the heat shock protein 70 (Hsp70) gene, respectively.

In addition to the above recombinant plasmids, pGEM-T Easy, used for cloning of PCR amplicons, and pET-41a-c (+), used for expression of the different gene fragments as GST fusion proteins in *E. coli*, were obtained from Promega and Novagen, respectively. The pET-41a-c (+) family of bacterial expression vectors are identical, except for the reading frame in which the cloned DNA fragment is expressed (Fig. 2.1). For plasmid DNA selection and maintenance in *E. coli*, the concentrations of antibiotics used were 100 µg/ml for ampicillin and 50 µg/ml for kanamycin. The antibiotics were obtained from Sigma-Aldrich.

2.2.2   Primers

Primers were designed based on the nucleotide sequences of the cloned insert DNA in pGEM-NS3Nterm, pACT2-SARA, Clone 2 and Clone 346, as provided by Dr. V. Van Staden and Mr W. Barnes. To facilitate cloning of the respective gene fragments into the pET-41a-c (+) family of bacterial expression vectors, the primers were designed to incorporate unique restriction enzyme recognition sites at the 5'-terminal ends. Primer pairs were analyzed for self-complementarity and primer-dimer formation, as well as for similar melting temperatures (T_m) using PerlPrimer v.1.1.20 (Marshall 2004). In the case of the truncated NS3 gene the forward primer was designed to incorporate a FLAG tag (Hopp *et al*. 1988; Einhauer and Jungbauer 2001), flanked by the recognition sequences of restriction enzymes required in subsequent cloning procedures. The primers, indicated in Table 2.1,

A

Xho I (174)
Eag I (182)
Not I (182)
Hind III (189)
Sal I (195)
Sac I (206)
Pst I (214)
Sse8387 I (214)
Asc I (216)
Stu I (226)
BsrG I (229)
EcoR I (235)
BamH I (241)
EcoR V (251)
Nco I (255)
PshA I (265)

Avr II(131)
Bpu1102 I(80)
Dra III(5691)

PinA I(292)
Kpn I(299)
Bgl II(302)
Mun I(360)
Sac II(393)
Spe I(424)
Swa I(667)

f1 origin (5474-5921)

GST·Tag (436-1095)

Msc I(887)

Sgf I(4990)
Pvu I(4990)

Kan (4559-5374)

Nde I(1094)
Xba I(1132)
SgrA I(1243)

Sma I(4864)

Nru I(4647)

Sph I(1399)

pET-41a(+)
(5933 bp)

Eco57 I(4336)

AlwN I(4204)

ori (3850)

lacI (157-1-2656)

BssS I(3961)

Mlu I(1924)

BstE II(2105)
Apa I(2135)

Bst1107 I(3559)
Tth111 I(3533)

Hpa I(2430)

Psp5 II(2794)

B

T7 promoter   lac operator   Xba I   rbs   Nde I
AATTAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGTCCCCT
                                                                                     Met Ser Pro

          GST·Tag   Spe I   His·Tag   Sac II
ATACTAGGTTAT...627bp...GACCATCCTCCAAAATCGGATGGTTCAACTAGTGGTTCTGGTCATCACCATCACCATCACTCCGCGGGTCTGGTGCCACGCGGTAGT
Ile Leu Gly Tyr...209aa...Asp His Pro Pro Lys Ser Asp Gly Ser Thr Ser Gly Ser Gly His His His His His His Ser Ala Gly Leu Val Pro Arg Gly Ser
                                                                                              thrombin

     Mun I   S·Tag   S·Tag primer #69945-3   Bgl II Kpn I   PinA I   PshA I
ACTGCAATTGGTATGAAAGAAACCGCTGCTGCTAAATTCGAACGCCAGCACATGGACAGCCCAGATCTGGGTACCGGTGGTGGCTCCGGTGATGACGACGACAAG
Thr Ala Ile Gly Met Lys Glu Thr Ala Ala Ala Lys Phe Glu Arg Gln His Met Asp Ser Pro Asp Leu Gly Thr Gly Gly Gly Ser Gly Asp Asp Asp Asp Lys
                                                                                                        enterokinase

     Nco I EcoR V BamH I EcoR I BsrG I Stu I   Asc I Pst I Sac I Sal I Hind III Eag I Xho I   His·Tag
                                                    Sse8387 I   Not I
AGTCCCATGGGATATCGGGGATCCGAATTCTGTACAGGCCTTGGCGCGCCTGCAGGCGAGCTCCGTCGACAAGCTTGCGGCCGCACTCGAGCACCACCACCACCACCACTAATTGATTAA   pET-41a(+)
Ser Pro Met Gly Tyr Arg Gly Ser Glu Phe Cys Thr Gly Leu Gly Ala Pro Ala Ala Glu Leu Arg Arg Gln Ala Cys Gly Arg Thr Arg Ala Pro Pro Pro Pro Pro Pro Pro Leu Ile Asp End

AGTCCCATGGATATCGGGGATCCGAATTCTGTACAGGCCTTGGCGCGCCTGCAGGCGAGCTCCGTCGACAAGCTTGCGGCCGCACTCGAGCACCACCACCACCACCACCACTAATTGATTAA   pET-41b(+)
Ser Pro Met Asp Ile Gly Asp Pro Asn Ser Val Gln Ala Leu Ala Arg Leu Gln Ala Ser Ser Val Asp Lys Leu Ala Ala Ala Leu Glu His His His His His His His His End

AGTCCCATGGCGATATCGGGGATCCGAATTCTGTACAGGCCTTGGCGCGCCTGCAGGCGAGCTCCGTCGACAAGCTTGCGGCCGCACTCGAGCACCACCACCACCACCACCACTAATTGATTAA   pET-41c(+)
Ser Pro Met Ala Ile Ser Gly Ile Arg Ile Leu Tyr Arg Pro Trp Arg Ala Cys Arg Arg Ala Pro Ser Thr Ser Leu Arg Pro His Ser Ser Thr Thr Thr Thr Thr Thr Thr Thr Asn End

     Avr II   Bpu1102 I   T7 terminator
TACCTAGGCTGCTAAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTG
                                        T7 terminator primer #69337-3

Figure 2.1: Vector map of the pET41a-c (+) expression vectors. **(A)** Vector map indicating the location of relevant restriction sites, as well as the location important genes and markers. **(B)** Representation of the different reading frames present in the pET41a-c (+) expression vectors.

were synthesized by Inqaba Biotechnical Industries.

Table 2.1: Primers used in this part of the study

Gene segment	Primer name	Incorporated restriction site	Nucleotide sequence*
SARA	SARA-fwd	*Bam*HI	5'-GC**ggatcc**AGGCCTGGAGTCAACACAAT-3'
	SARA-rev	*Hind*III	5'-GC**aagctt**CACCGTTTAATGTGGGATATCACT-3'
Hsp70	Hsp-fwd	*Bam*HI	5'-GC**ggatcc**ATTCGCGGCCGCGTC-3'
	Hsp-rev	*Hind*III	5'-CGC**aagctt**GCTTAGTCGACCTCCTCGAC-3'
UB	UB-fwd	*Bam*HI	5'-**ggatcc**GCGGCCGCGTCGACCTCA-3'
	UB-rev	*Hind*III	5'-GC**aagctt**ACATTGAACACACAAATTGAAAATTTTATT-3'
NS3	FLAG-NS3-fwd[#]	*Nde*I and *Hind*III	5'-G**catatg**GATTACAAGGATGACGATGAC**aagctt**ATGAGTCTAGCTACGATCGCC-3'
	NS3-rev	*Xho*I	5'-C**GCTCGAG**TTACATTGACGCCAACTC-3'

* The restriction endonuclease sites are indicated in bold lower case letters
[#] A FLAG tag sequence incorporated at the 5' end of the truncated NS3 gene is underlined

2.2.3 PCR amplification

The PCR reactions were prepared on ice in 0.2-ml Eppendorf tubes and contained 50 ng of template DNA (either pACT2-SARA, pGEM-NS3Nterm, Clone 2 or Clone 346), 10 pmol each of the appropriate forward and reverse primer (Table 2.1), 200 µM of each deoxynucleotide triphosphate (dNTP), $1 \times$ Reaction buffer, 1.5 mM MgCl$_2$, 1 U of SuperTherm *Taq* polymerase (Seperation Scientific), and nuclease-free water to a final volume of 25 µl. A negative control reaction was prepared in a similar manner, except that template DNA was omitted. The PCR tubes were placed in an Eppendorf Mastercycler® Gradient thermal cycler. Following incubation at 94°C for 2 min, the samples were subjected to 25 cycles of denaturation at 94°C for 30 s, primer annealing at 62°C for 30 s and elongation at 72°C for 1 min. After the last cycle, a final extension step was performed at 72°C for 3 min.

2.2.4 Agarose gel electrophoresis

The PCR products were analyzed by agarose gel electrophoresis (Sambrook and Russell 2001). To this end, either 1% or 2% (w/v) agarose gels were prepared by melting SeaKem® LE agarose (Lonza) in $1 \times$ TAE buffer (40 mM Tris-HCl, 20 mM NaOAc, 1 mM EDTA; pH 8.5) and casting horizontal gels. To allow visualization of the DNA when viewed on a UV transilluminator, the gels were supplemented with 0.5 µl of ethidium bromide (10 mg/ml). In addition to the samples, a DNA molecular weight marker was also loaded to determine the

size of the PCR products. The agarose gels were electrophoresed at 90 V for approximately 45 min, before being visualized on the UV transilluminator.

2.2.5   Purification of DNA fragments from agarose gels

The PCR products were purified from agarose gel slices with the Zymoclean Gel DNA Recovery kit (Zymo Research Corporation) according to the manufacturer's instructions. Briefly, the appropriate DNA fragment was excised from the gel using a scalpel blade and 3 volumes of the supplied ADB buffer was added to each gel slice. Following incubation at 50°C for 5-10 min, the melted agarose solutions were centrifuged through Zymo-Spin™ DNA-binding columns at 13 400 rpm for 1 min. Each column was subsequently washed twice with 200 µl of Wash buffer and the DNA was eluted in 10 µl of nuclease-free water. An aliquot of each sample was analyzed by agarose gel electrophoresis to assess the purity and concentration of the DNA.

2.2.6   Ligation of purified PCR products into pGEM-T Easy

The purified PCR products were cloned by T/A cloning (Holton and Graham 1990) with the pGEM-T Easy Vector System (Promega). Each ligation reaction contained 50 ng of purified PCR product, 50 ng of pGEM-T Easy vector DNA, 5 µl of $2 \times$ Rapid Ligation buffer, 1 µl of T4 DNA ligase (3 Weiss units/µl) and nuclease-free water to a final volume of 10 µl. The reactions were incubated overnight at 4°C.

2.2.7   Preparation and transformation of competent *E. coli* JM109 cells

Competent *E. coli* JM109 cells were prepared with the calcium chloride-method (Sambrook and Russell 2001). The *E. coli* JM109 culture was first streaked from a frozen glycerol stock onto M9 minimal medium agar (90 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.5 mM NaCl, 18.6 mM NH_4Cl, 11.1 mM glucose, 2 mM $MgSO_4$, 0.01 mM $CaCl_2$, 0.033 mM thiamine, 0.035 mM $FeSO_4$, 1.2 g bacteriological agar; pH 7.4) and incubated at 37°C until visible bacterial colonies appeared (approximately two days). A single colony was subsequently inoculated into 50 ml of sterile LB broth and incubated at 37°C until an OD_{600} of 0.7 was obtained. The culture was then placed on ice for 30 min to inhibit further bacterial growth. The bacterial cells were harvested by centrifugation at 5000 rpm for 8 min at 4°C. The cell pellet was suspended in 10 ml of an ice-cold 80 mM $CaCl_2$-50 mM $MgCl_2$ solution, followed by incubation on ice for 10 min. The centrifugation step was repeated and the cells were

suspended in 10 ml of ice-cold 100 mM CaCl$_2$. Glycerol was added to the cell suspension (50% [v/v]) and 200-µl aliquots were pipetted into 1.5-ml Eppendorf tubes before being flash-frozen in liquid nitrogen. The samples were used immediately or stored at -70°C.

To transform the competent *E. coli* JM109 cells, the entire ligation reaction was added to the competent cells (200 µl) and incubated on ice for 30 min. Following incubation, the tubes were subjected to a heat shock at 42°C for 90 s and then immediately chilled on ice for 2 min. Subsequently, 800 µl of pre-warmed (37°C) LB broth was added to the tubes, followed by incubation at 37°C for 1 h to allow expression of ampicillin resistance encoded by the pGEM-T Easy vector. To allow for blue/white screening, the cells were plated together with 50 µl of 0.1 M IPTG and 20 µl of 50 mg/ml X-gal onto LB agar supplemented with ampicillin. The agar pates were incubated overnight at 37°C. As controls, competent cells were transformed with plasmid DNA of a known concentration to determine the transformation efficiency, and untransformed cells were used to test for contamination.

2.2.8   Plasmid DNA extractions

Plasmid DNA was extracted from selected colonies by the alkaline lysis method (Birnboim and Doly 1979), as described by Sambrook and Russell (2001). Single colonies were inoculated into 5 ml of LB broth containing ampicillin and incubated overnight at 37°C. The cells from 1 ml of the overnight cultures were harvested by centrifugation at 5000 rpm for 5 min. The cell pellets were suspended in 100 µl of ice-cold Solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris; pH 8) and incubated at room temperature for 5 min. The cells were lysed by addition of 200 µl of freshly prepared Solution II (0.2 N NaOH, 1% [w/v] SDS) and after incubation on ice for 5 min, 150 µl of ice-cold Solution III (3 M NaOAc; pH 4.8) was added. Following incubation on ice for 5 min, the insoluble aggregate that formed was removed by centrifugation at 13 400 rpm for 5 min. The plasmid DNA was precipitated from the recovered supernatants by addition of 2 volumes of absolute ethanol and incubation on ice for 30 min. The precipitated plasmid DNA was pelleted by centrifugation at 13 400 rpm for 15 min, rinsed twice with 70% ethanol, air-dried and then suspended in 20 µl of nuclease-free water. Contaminating RNA was degraded by addition of 1 µl of RNase A (10 mg/ml), followed by incubation at 37°C for 30 min. The plasmid DNA was analyzed by electrophoresis on a 1% (w/v) agarose gel. When larger amounts of plasmid DNA were required, multiple extractions were performed from the same overnight bacterial culture and the samples pooled.

2.2.9 Restriction enzyme digestion

Plasmid DNA was characterized by restriction enzyme digestion. The digestion reactions were performed in 1.5-ml Eppendorf tubes and contained 1 µg of plasmid DNA, 10 U of *Eco*RI, 2.5 µl of $10 \times$ SuRE/Cut Buffer H (Roche) and nuclease-free water to a final volume of 25 µl. The tubes were incubated at 37°C for 2 h, after which the restriction enzyme was inactivated by incubation at 65°C for 15 min. The digestion products were analyzed by agarose gel electrophoresis in the presence of a DNA molecular weight marker.

2.2.10 Sequencing of cloned insert DNA

The nucleotide sequence of cloned insert DNA was determined with the ABI-PRISM® BigDye™ Terminator v.3.1 Cycle Sequencing Ready Reaction kit (Applied Biosystems) according to the manufacturer's instructions. Each sequencing reaction contained 500 ng of recombinant plasmid DNA, 2 µl of BigDye Ready Reaction Mix, 1 µl of $5 \times$ BigDye Sequencing Buffer, 3.2 pmol of M13 forward primer (5'-CCCAGTCACGACGTTGTAAAACG-3') and nuclease-free water to a final volume of 10 µl. The tubes were placed in an Eppendorf Mastercycler® Gradient thermal cycler. Following initial denaturation at 94°C for 1 min, the reaction mixtures were subjected to 25 cycles of denaturation at 94°C for 10 s, primer annealing at 50°C for 15 s and elongation at 60°C for 4 min. The extension products were precipitated by addition of 11 µl of nuclease-free water, 2 µl of 3 M NaOAc (pH 4.6) and 60 µl of ice-cold absolute ethanol. The tubes were incubated on ice for 15 min and subsequently centrifuged at 13 400 rpm for 30 min. The supernatant was carefully discarded and the pellet was rinsed twice with 70% ethanol. The samples were submitted to the University of Pretoria's Sequencing Facility for sequencing on an ABI-PRISM® 3130XL DNA sequencer. The nucleotide sequences obtained were analyzed with the BioEdit v.7.0.9.0 software program (Hall 1999).

2.2.11 Construction of recombinant pET-41a-c (+) bacterial expression vectors

- *Preparation of vector and insert DNA*

The cloned SARA, Hsp70 and UB gene fragments were recovered from the recombinant pGEM-T Easy plasmids by digestion with both *Bam*HI and *Hind*III, and cloned into identically digested pET-41a (+), pET-41b (+) and pET-41c (+) vector DNA. The reaction mixtures contained 1 µg of plasmid DNA, 10 U of each *Bam*HI and *Hind*III, 2.5 µl of $10 \times$

SuRE/Cut Buffer B and nuclease-free water to a final volume of 25 µl. The tubes were incubated at 37°C for 2 h and the restriction enzymes were then inactivated by incubation at 65°C for 15 min. To enable cloning of the FLAG-tagged truncated NS3 gene fragment into pET-41a (+) vector DNA, the respective plasmid DNAs were digested with both *Nde*I and *Xho*I. The digestion reactions were performed as described above, except that Buffer O was used and the restriction enzymes were inactivated by incubation at 80°C for 20 min. All restriction enzymes were obtained from Roche and Thermo Scientific. The digestion products were analyzed on a 1% (w/v) agarose gel in the presence of a DNA molecular weight marker. The appropriate insert and vector DNA fragments were excised from the agarose gel and purified with the Zymoclean Gel DNA Recovery kit (Zymo Research Corporation), prior to their use in ligation reactions.

- *Ligation reactions*

Ligation of the purified DNA fragments and pET-41a-c (+) vector DNA was performed in 20-µl reaction volumes that each contained 50 ng of insert DNA, 50 ng of vector DNA, 1 × T4 DNA ligase buffer (66 mM Tris-HCl [pH 7.6], 6.6 mM $MgCl_2$, 10 mM DTT, 0.1 mM ATP) and 1 µl of T4 DNA ligase (350 U/µl; Takara Bio, Inc.). The ligation reactions were incubated overnight at 16°C. The entire ligation reaction was then transformed into competent *E. coli* JM109 cells, plated onto LB agar supplemented with kanamycin (50 µg/ml) and the agar plates were incubated overnight at 37°C. The integrity of the derived recombinant plasmid constructs were confirmed by agarose gel electrophoresis and restriction enzyme digestions.

2.2.12 Expression of recombinant proteins in *E. coli*

Competent cells of the *E. coli* BL21(DE3), *E. coli* BL21-Star(DE3) and *E. coli* BL21-Gold(DE3) strains were prepared and then transformed with recombinant pET-41a-c (+) expression plasmids, as described previously (Section 2.2.7), except that the cells were plated onto LB agar supplemented with kanamycin. Single colonies resulting from each transformation were inoculated into 20 ml of LB broth containing kanamycin and incubated overnight at 37°C with shaking. Expression of the recombinant GST-tagged proteins was induced with IPTG, as described by Studier *et al.* (1986 and 1990). A 5-ml aliquot of the overnight cultures was diluted 1:10 in 50 ml of sterile LB broth and incubated at 37°C for 2 h. To each culture, IPTG was added to a final concentration of 0.1 mM and the cultures were

incubated at 37°C for an additional 6 h. The bacterial cells were then harvested by centrifugation at 5000 rpm for 8 min at 4°C. The cell pellets were washed with $1 \times$ phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.2H_2O$, 1.4 mM KH_2PO_4; pH 7.4) and suspended in 5 ml of the same buffer.

2.2.13   SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

An aliquot of each protein sample was mixed with an equal volume of $2 \times$ protein solvent buffer (PSB: 125 mM Tris [pH 6.8], 4% [w/v] SDS, 20% [v/v] glycerol, 10% [v/v] β-mercaptoethanol, 0.001% [w/v] bromophenol blue). The samples were heated at 90°C for 10 min to ensure denaturation of the proteins. The proteins were subsequently resolved by electrophoresis in a discontinuous gel system, as described by Laemmli (1970). A 5% acrylamide stacking gel and 12% acrylamide separating gel were used, of which the acrylamide:bisacrylamide ratio was 30:0.8. The low porosity separating gel (0.375 mM Tris-HCl [pH 8.8], 0.1% [w/v] SDS) and high porosity stacking gel (0.125 M Tris-HCl [pH 6.8], 0.1% [w/v] SDS) were each polymerized by addition of 100 μl of 10% (w/v) ammonium persulphate and 10 μl of TEMED. Electrophoresis was performed in a Hoefer miniVE™ electrophoresis unit at 100 V for 2-3 h in $1 \times$ TGS buffer (25 mM Tris-HCl [pH 8.3], 192 mM glycine, 0.1% [w/v] SDS; pH 8.3). Following electrophoresis, the gels were stained for 15 min in Coomassie brilliant blue (0.3% [w/v] Coomassie brilliant blue, 50% [v/v] methanol, 10% [v/v] acetic acid) and then destained overnight in a solution containing 10% (v/v) methanol and 10% (v/v) acetic acid. In addition to the protein samples, a protein molecular mass marker was also loaded in order to determine the sizes of the recombinant proteins.

2.2.14   Western blotting

After SDS-PAGE, an unstained gel and Hybond™-C+ nitrocellulose membrane (GE Healthcare), cut to the same size as the gel, were equilibrated for 30 min in transfer buffer (25 mM Tris, 192 mM glycine) (Sambrook and Russell 2001). The proteins were transferred from the gel onto the nitrocellulose membrane at 6-8 V/cm for 2-3 h with a Mighty Small™ Transphor electrophoresis unit (Hoefer). Following transfer, the membrane was rinsed once for 5 min in $1 \times$ PBS, after which non-specific binding sites were blocked by incubating the membrane overnight in blocking solution (1% [w/v] fat-free milk powder in $1 \times$ PBS). The membrane was transferred to $1 \times$ PBS containing the primary antibody. These comprised of a

polyclonal anti-GST antibody (Santa Cruz Biotechnology) and either an anti-NS3 antiserum or anti-βgal-NS3 antiserum (both provided by Dr. V. Van Staden), which were diluted 1:1000 and 1:300 in $1 \times$ PBS, respectively. Following incubation at room temperature for 2 h with gentle agitation, the unbound primary antibody was removed by washing the membrane three times for 5 min each with wash buffer (0.05% [v/v] Tween-20 in $1 \times$ PBS). The secondary antibody, Protein-A conjugated to horseradish peroxidase (Sigma-Aldrich) diluted 1:1000 in $1 \times$ PBS, was added to the membrane and incubated at room temperature for 1 h. The membrane was then washed three times for 5 min each with wash buffer and once with $1 \times$ PBS for 5 min. The antigen-antibody complexes were detected calorimetrically by immersing the membrane in peroxidase enzyme substrate (60 mg of 4-chloro-1-naphtol in 20 ml ice-cold methanol and 100 ml of $1 \times$ PBS containing 60 µl H_2O_2, mixed just prior to use). Once bands became visible, the membrane was rinsed with distilled water and air-dried.

2.2.15   Immobilization of GST fusion proteins to glutathione agarose beads

The GST fusion proteins (SARA, Hsp70 and UB) were expressed, as described above (Section 2.2.12), except that 10-ml cultures of the appropriate recombinant *E. coli* strains were induced with IPTG. The bacterial cells were harvested by centrifugation at 5000 rpm for 8 min at 4°C and then lysed according to the procedures described by Theron *et al.* (1994). The cell pellets of bacterial cultures expressing the GST fusion proteins were suspended in 1 ml of ice-cold Lysis buffer (20% [w/v] sucrose, 100 mM Tris-HCl [pH 8.0], 10 mM EDTA). Subsequently, 1 µl of lysozyme (5 mg/ml) was added to each of the samples and the samples were incubated at room temperature for 15 min and then on ice for 2 h. Following addition of DTT to 10 mM, TritonX-100 and Tween-20 both to 1% (v/v), the samples were incubated on ice for a further 30 min. Finally, the cell lysates were treated with 1 µl of RNase A (10 mg/ml) and 1 µl of DNase (1 U/µl) for 20 min at room temperature to degrade the nucleic acids. The cell lysates were clarified by centrifugation (13 400 rpm, 15 min) and the supernatant, considered the soluble protein fraction, was recovered. The pellet (insoluble protein fraction) was suspended in 500 µl of $1 \times$ PBS. Both the soluble and insoluble protein fractions were analyzed by SDS-PAGE and Western blot analyses, as described previously (Sections 2.2.13 and 2.2.14).

The GST fusion proteins were immobilized onto glutathione agarose beads for subsequent use in pull-down assays. The glutathione agarose (sulphur linkage; Sigma-Aldrich) was prepared according to the manufacturer's instructions. Briefly, the glutathione agarose was

pre-swollen in distilled water overnight at 4°C, washed and then suspended in $1 \times$ PBS as a 50% slurry. Whole-cell lysates or clarified supernatant fractions containing soluble GST fusion proteins (1 ml) were mixed with 100 µl of the slurry and incubated at room temperature for 45 min with gentle agitation to allow adsorption of the GST fusion proteins to the glutathione agarose beads. After incubation, the glutathione agarose beads were collected by centrifugation at 3500 rpm for 5 min and washed three times with 1 ml of $1 \times$ PBS. The washed beads were subsequently suspended in 50 µl of either TENT buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA, 1% [v/v] Triton X-100) or NP-40 buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% [v/v] Nonidet P-40). Immobilization of the GST fusion proteins to the glutathione agarose was evaluated by SDS-PAGE and Western blot analysis using a polyclonal anti-GST antibody.

2.2.16   Pull-down assays

For pull-down experiments, bacterial cultures expressing the FLAG-tagged truncated AHSV NS3 protein were induced as described previously (Section 2.2.12). Following induction, the bacterial cells were harvested and lysed in 1 ml of either TENT buffer or NP-40 buffer. For pull-down assays, performed essentially as described by Wirblich *et al.* (2006), aliquots (400 µl) of whole-cell lysates containing the FLAG-tagged truncated NS3 protein was mixed with 40 µl of the respective immobilized GST fusion proteins, and incubated at room temperature for 2 h on a shaking platform. The glutathione agarose beads were collected by centrifugation at 1500 rpm for 5 min and washed three times with 1 ml of the lysis buffer used in the assay (either TENT of NP-40). After the final wash, the glutathione agarose beads were suspended in 20 µl of the appropriate buffer (either TENT of NP-40). These samples, as well as aliquots of the whole-cell lysate (20 µl) removed after incubation, were mixed with an equal volume of $2 \times$ PSB, heated at 90°C for 10 min and then loaded directly onto a SDS-polyacrylamide gel. Following SDS-PAGE, the unstained gels were subjected to Western blot analysis using an anti-NS3 antiserum.

2.3 RESULTS

2.3.1 Construction of recombinant pET41a-c (+) expression plasmids harbouring different insect gene fragments

Towards constructing recombinant plasmids that would allow expression of the NS3-interacting SARA, Hsp70 and UB peptides as GST fusion proteins for use in pull-down experiments, the respective gene fragments first had to be prepared for directional cloning into the pET41a-c (+) family of bacterial expression vectors. For the cloned gene fragments to be expressed correctly they have to be in the correct translational reading frame. This can be ensured by cloning the gene fragments into the three expression vectors (a-c), since they each contain identical cloning sites but in a different reading frame (see Fig. 2.1).

To facilitate cloning procedures the SARA, Hsp70 and UB gene fragments were PCR-amplified using primers that were extended at their 5' ends by additional nucleotides to incorporate unique restriction endonuclease recognition sites. The respective gene fragments were thus PCR-amplified using recombinant plasmids pACT-SARA, Clone 2 and Clone 346 as template DNA, together with the appropriate forward primers (containing a *BamHI* site) and reverse primers (containing a *Hind*III site). The amplicons, approximately 184 bp for SARA, 677 bp for Hsp70 and 639 bp for UB, were each cloned into pGEM-T Easy vector DNA. Following transformation of competent *E. coli* JM109 cells, recombinant transformants with a white colony phenotype were selected from X-gal–containing indicator plates and cultured overnight in LB broth supplemented with ampicillin. The derived recombinant constructs, designated pGEM-SARA, pGEM-Hsp70 and pGEM-UB, respectively, were analyzed for the presence of a cloned insert DNA by digestion with *Eco*RI that flanks the insertion site of the pGEM-T Easy vector DNA. Digestion of the recombinant plasmid DNA resulted in the excision of the cloned SARA, Hsp70 and UB insert DNAs (Fig. 2.2, lanes 2 to 4). As a final confirmation regarding the integrity of the cloned insert DNA, the nucleotide sequence of each of the cloned insert DNAs was determined. No nucleotide differences were observed between the sequences of these gene fragments and those supplied previously (Dr. V. Van Staden and Mr W. Barnes, unpublished). The nucleotide sequences of the cloned gene fragments are provided in the Appendix to this dissertation.

The above recombinant plasmids were subsequently used as sources in the construction of the desired bacterial expression vectors. For this purpose, the SARA, Hsp70 and UB gene
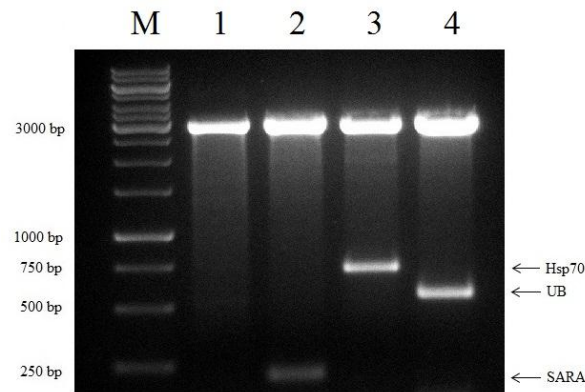
Figure 2.2: Agarose gel electrophoretic analysis of recombinant pGEM-T Easy constructs harbouring different gene fragments, following digestion with *Eco*RI. Samples were loaded as follows: lane M – GeneRuler 1 kb DNA ladder; lane 1 – *Eco*RI digest of non-recombinant pGEM-T Easy; lane 2 – *Eco*RI digest of pGEM-SARA; lane 3 – *Eco*RI digest of pGEM-Hsp70; lane 4 – *Eco*RI digest of pGEM-UB.
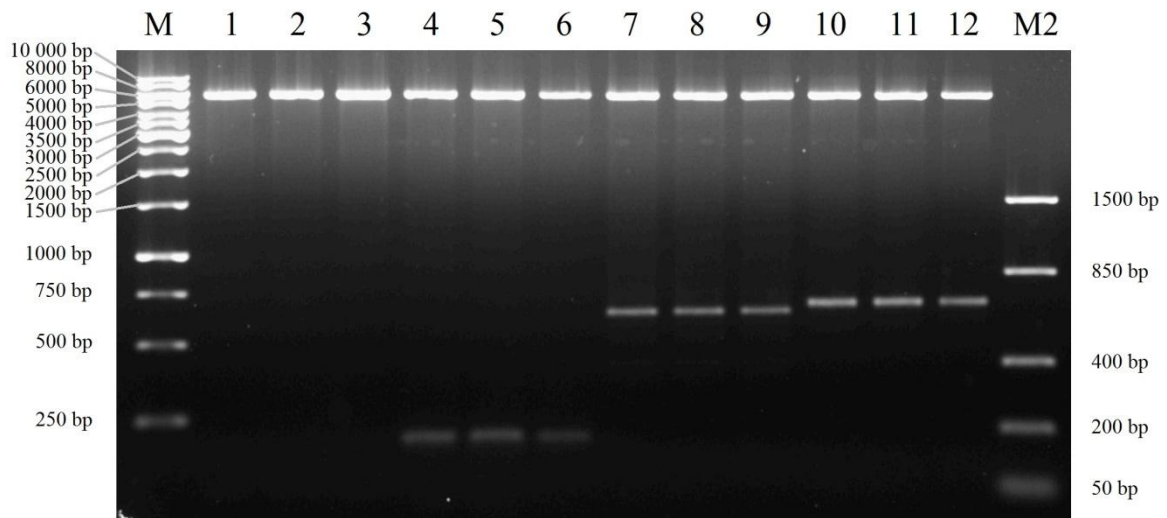
Figure 2.3: Agarose gel electrophoretic analysis of recombinant pET41a-c (+) bacterial expression plasmids, following digestion with both *Bam*HI and *Hind*III. Samples were loaded as follows: lane M – GeneRuler 1kb DNA ladder; lane 1 – pET 41a (+); lane 2 – pET41b (+); lane 3 – pET41c (+); lane 4 – pET41a-SARA; lane 5 – pET41b-SARA; lane 6 – pET41c-SARA; lane 7 – pET41a-UB; lane 8 – pET41b-UB; lane 9 – pET41c-UB; lane 10 – pET41a-Hsp70; lane 11 – pET41b-Hsp70; lane 12 – pET41c-Hsp70; lane M2 – FastRuler low range DNA ladder.

fragments were recovered from the respective recombinant pGEM-T Easy constructs by digestion with both *Hind*III and *Bam*HI. The agarose gel-purified DNA fragments were each cloned into identically prepared pET41a (+), pET41b (+) and pET41c (+) vector DNA. Since the pET41a-c (+) expression vectors do not allow for blue/white selection, plasmid DNA was extracted from randomly selected kanamycin-resistant transformants and then characterized by agarose gel electrophoresis and restriction enzyme digestion. Plasmid DNA migrating slower than the parental pET41a-c (+) vector DNA were selected and analyzed for the presence of a cloned insert DNA by digestion with both *Hind*III and *Bam*HI. In contrast to the non-recombinant plasmid DNA, which was linearized, digestion of recombinant plasmid DNA excised DNA fragments corresponding in size to the SARA (184 bp; Fig. 2.3, lanes 4-6), UB (639 bp; Fig. 2.3, lanes 7-9), and Hsp70 (677 bp; Fig. 2.3, lanes 10-12) gene fragments. These results therefore confirmed that the respective gene fragments were cloned successfully into each of the pET41a-c (+) bacterial expression vectors.

The nine recombinant plasmids were designated pET41a-SARA, pET41b-SARA, pET41c-SARA, pET41a-Hsp70, pET41b-Hsp70, pET41c-Hsp70, pET41a-UB, pET41b-UB and pET41c-UB, respectively. These recombinant plasmids were subsequently evaluated for their ability to express the SARA, Hsp70 and UB peptides as GST fusion proteins, as described below.

2.3.2 Expression of recombinant GST fusion proteins

To determine which of the constructed pET41a-c (+) plasmids are capable of expressing the SARA, UB and Hsp70 peptides as GST fusion proteins in *E. coli*, the non-recombinant pET-41a (+) and nine recombinant pET41a-c (+) expression plasmids were transformed into competent *E. coli* BL21(DE3) cells. For bacterial expression, overnight cultures of the recombinant *E. coli* BL21(DE3) strains were diluted in fresh LB broth and grown in the presence of IPTG for 6 h to induce high-level protein expression. Following incubation, whole-cell lysates were prepared of the respective bacterial cultures and analyzed by SDS-PAGE.

Analysis of the Coomassie blue-stained gels indicated the presence of an apparently novel protein in samples prepared from the *E. coli* BL21(DE3) cells transformed with the pET41c-SARA (Fig. 2.4A, lane 6), pET41c-UB (Fig. 2.4B, lane 6) and pET41a-Hsp70 (Fig. 2.4B,

lane 7) bacterial expression plasmids. The molecular mass of these novel proteins was in agreement with the predicted molecular mass of the GST protein (31.7 kDa) fused in-frame with the 5.5-kDa SARA peptide, 16.9-kDa UB peptide or 23.8-kDa Hsp70 peptide. In contrast to cells transformed with these specific recombinant plasmids, induced bacterial cultures transformed with the remainder of the recombinant pET41 plasmids expressed proteins that were either similar in size or slightly smaller than the GST protein expressed in *E. coli* BL21(DE3) cells transformed with the non-recombinant pET-41a (+) vector. These proteins most likely represent early termination products as a consequence of out-of-frame translation. To confirm the identity of the novel GST-SARA (37.2 kDa), GST-UB (48.6 kDa) and GST-Hsp70 (55.5 kDa) fusion proteins, an unstained SDS-polyacrylamide gel was subjected to Western blot analysis, the results of which indicated that these proteins were recognized by the polyclonal anti-GST antibody (results not shown, see Fig. 2.5B, lanes 2, 5 and 6).

2.3.3 Purification of recombinant GST fusion proteins by immobilization onto glutathione agarose beads

To determine whether the GST fusion proteins were expressed in a soluble form, the cells of IPTG-induced recombinant *E. coli* BL21(DE3) cultures were harvested and soluble and insoluble protein fractions were prepared. The samples were analyzed by SDS-PAGE. The results indicated that the GST-UB and GST-Hsp70 fusion proteins could be detected in the soluble cytoplasmic protein fraction, whereas the GST-SARA fusion protein was detected almost exclusively in the insoluble protein fraction (results not shown). Consequently, in an effort to obtain soluble GST-SARA, the recombinant pET41c-SARA plasmid was transformed into competent *E. coli* BL21-Star(DE3) and *E. coli* BL21-Gold(DE3) cells and expression of the GST-SARA fusion protein was induced with IPTG.

The GST-SARA fusion protein was purified directly from the whole-cell lysates using glutathione affinity chromatography in order to determine whether it was expressed in a soluble form in the two different *E. coli* strains. For this purpose, the whole-cell lysates were incubated with glutathione agarose, and the agarose beads were then collected by centrifugation and loaded onto a SDS-polyacrylamide gel. The soluble GST-UB and GST-Hsp70 fusion proteins, expressed in *E. coli* BL21(DE3) cells, were included as controls in this assay. The results indicated that although the GST-SARA fusion protein was over-expressed in the respective bacterial strains, albeit to differing levels, it could only be purified
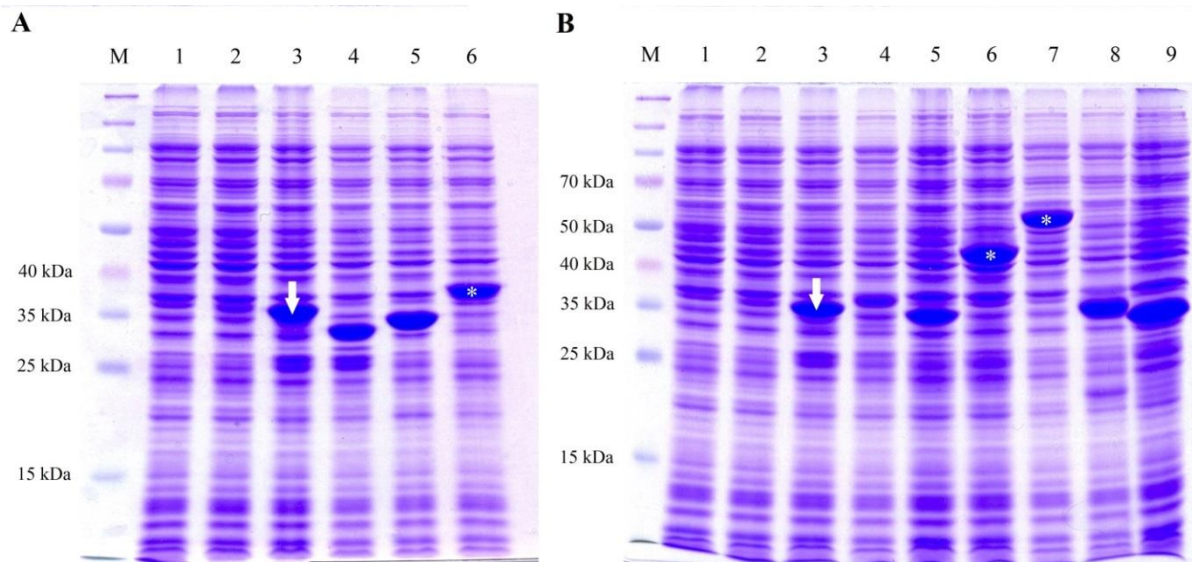
Figure 2.4: SDS-PAGE analysis of GST fusion proteins expressed in *E. coli* BL21(DE3). Whole-cell lysates were prepared at 6 h post-induction of IPTG-induced cultures of *E. coli* BL21(DE3) cells transformed with the recombinant pET41a-c (+) expression plasmids. Untransformed *E. coli* BL21(DE3) cells and *E. coli* BL21(DE3) cells transformed with the non-recombinant pET41a (+) plasmid were included as controls. **(A)** SDS-polyacrylamide gel indicating expression of the GST-SARA fusion protein. The gel was loaded as follows: lane M – Spectra Multicolor Broad Range Protein Ladder (Thermo Scientific); lane 1 – Untransformed *E. coli* BL21(DE3); lane 2 – Uninduced *E. coli* BL21(DE3) transformed with pET41a (+); lane 3 – Induced *E. coli* BL21(DE3) transformed with pET41a (+); lanes 4 to 6 – Induced *E. coli* BL21(DE3) transformed with pET41a-SARA; pET41b-SARA and pET41c-SARA, respectively. **(B)** SDS-polyacrylamide gel indicating expression of the GST-Hsp70 and GST-UB fusion proteins. The gel was loaded as follows: lane M, as well as lanes 1-3 were loaded as described for (A); lanes 4 to 6 – Induced *E. coli* BL21(DE3) transformed with pET41a-UB, pET41b-UB and pET41c-UB, respectively; lanes 7 to 9 – Induced *E. coli* BL21(DE3) transformed with pET41a-Hsp70, pET41b-Hsp70 and pET41c-Hsp70, respectively. The GST protein is indicated with an arrow, whereas the novel GST-SARA, GST-Hsp70 and GST-UB fusion proteins are indicated with asterisks.

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successfully from the whole-cell lysate of the recombinant *E. coli* BL21-Star(DE3) strain. In contrast to the GST-UB and GST-Hsp70 fusion proteins, which could be purified in large amounts, the GST-SARA protein was purified in comparatively much lower amounts (Fig. 2.5A, lanes 2, 5 and 6, respectively). These results thus suggest that the GST-SARA fusion protein is expressed in a predominantly insoluble form.

The identity of the matrix-immobilized GST fusion proteins were subsequently verified by subjecting an unstained SDS-polyacrylamide gel to Western blot analysis using a polyclonal anti-GST antibody (Fig. 2.5B). The antibody reacted with the GST-SARA, GST-UB and GST-Hsp70 fusion proteins, indicating that these GST fusion proteins could be immobilized onto glutathione agarose beads and therefore be used in subsequent pull-down experiments.

2.3.4 Construction of recombinant plasmid pET41a-FNS3

Since the truncated AHSV NS3 protein was to be used in pull-down experiments with different GST fusion proteins, it was imperative that it lacks a GST tag moiety so as to avoid false positive results. Consequently, a cloning strategy was designed that would result in deletion of the GST coding sequence from the bacterial expression vector and therefore enable expression of a non-GST tagged polypeptide. In addition, the truncated NS3 protein was modified through the incorporation of a FLAG tag at its N-terminus. This was done with the aim of performing co-immunoprecipitation assays as an alternative to pull-down assays for verifying the NS3-insect cell protein interactions. The cloning strategy that was followed and the results obtained are indicated below.

Using plasmid pGEM-NS3Nterm as template DNA, PCR amplification was performed using primers FLAG-NS3-fwd (containing an *Nde*I site) and NS3-rev (containing an *Xho*I site), as described under Materials and Methods (Section 2.2.3). An aliquot of the reaction mixture was analyzed by agarose gel electrophoresis and a single amplicon of the expected size (366 bp) was observed. Using a similar cloning and screening strategy to that described previously, the amplicon was purified from the agarose gel and cloned into pGEM-T Easy to generate pGEM-FNS3. Digestion of the recombinant plasmid DNA with *Eco*RI yielded restriction fragments of approximately 3.0 kb and 366 bp, which is in agreement with the expected size of the pGEM-T Easy vector DNA and insert DNA, respectively (Fig. 2.6, lane 2). The integrity of the cloned insert DNA was verified by nucleotide sequencing prior to it being used in subsequent cloning procedures. The nucleotide sequence is provided in the Appendix to this dissertation.
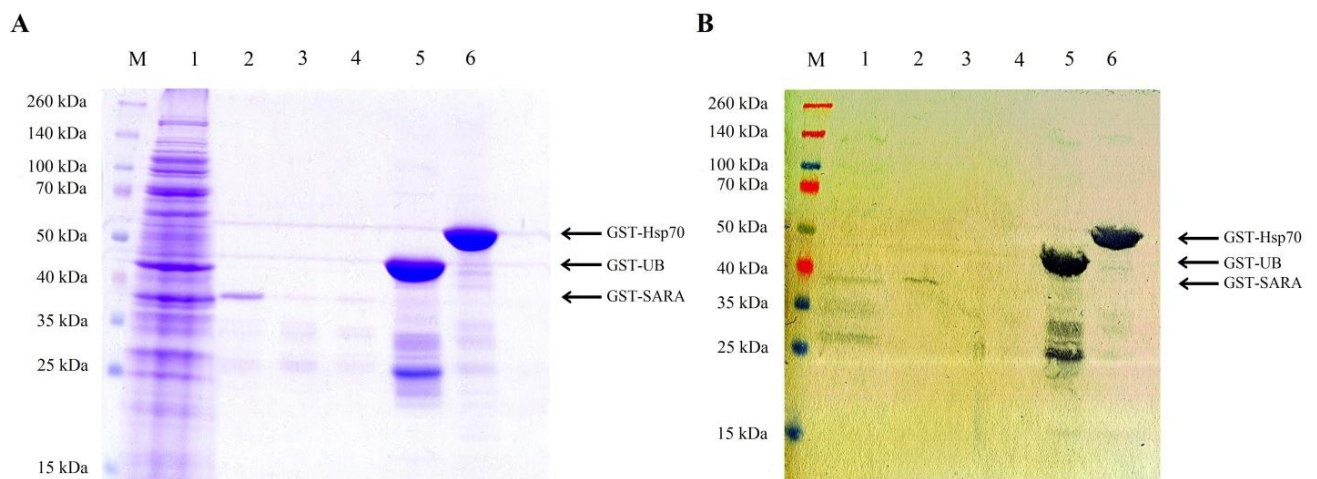
Figure 2.5: Expression and purification of the GST-SARA, GST-UB and GST-Hsp70 fusion proteins. Following expression of these GST fusion proteins in IPTG-induced *E. coli* cultures, bacterial lysates were prepared, incubated with glutathione agarose and the glutathione agarose beads were then collected by centrifugation. GST fusion proteins immobilized onto the glutathione agarose beads were resolved by SDS-PAGE **(A)** and a duplicate unstained gel was subjected to Western blot analysis with a polyclonal anti-GST antibody **(B)**. For both **(A)** and **(B)**, samples were loaded as follows: lane M – Spectra Multicolor Broad Range Protein Ladder (Thermo Scientific); lane 1 – Whole-cell lysate of *E. coli* BL21-Star(DE3) expressing the GST-SARA fusion protein; lane 2 – Immobilized GST-SARA fusion protein from the whole-cell lysate of a recombinant *E. coli* BL21-Star(DE3) culture; lane 3 – Immobilized GST-SARA fusion protein from the soluble fraction of a recombinant *E. coli* BL21-Gold(DE3) culture; lane 4 – Immobilized GST-SARA fusion protein from the whole-cell lysate of a recombinant *E. coli* BL21(DE3) culture; lane 5 – Immobilized GST-UB fusion protein from the soluble fraction of a recombinant *E. coli* BL21(DE3) culture; lane 6 – Immobilized GST-Hsp70 fusion protein from the soluble fraction of a recombinant *E. coli* BL21(DE3) culture.

The modified truncated AHSV NS3 gene was subsequently cloned directionally into the pET41a (+) expression vector to position it in the correct translational reading frame for expression. To construct recombinant plasmid pET41a-FNS3, the FNS3 gene was recovered from pGEM-FNS3 by digestion with both *Nde*I and *Xho*I, purified from an agarose gel and then cloned into pET41a (+) vector DNA, which was likewise prepared and thus resulted in the deletion of the GST coding sequence. Following transformation of competent *E. coli* JM109 cells with the ligation reaction mixture, the plasmid DNA extracted from kanamycin-resistant transformants was characterized by agarose gel electrophoresis and by restriction endonuclease digestion. Digestion of the recombinant plasmid DNA with both *Nde*I and *Xho*I yielded DNA fragments corresponding in size to the vector DNA lacking the GST coding sequence (4.97 kb) and the modified truncated NS3 gene (366 bp) (Fig. 2.7, lane 2), thereby indicating that that the gene had been cloned successfully. The recombinant pET41a-FNS3 plasmid was used in all subsequent experiments.

2.3.5 Expression of a truncated AHSV NS3 protein in *E. coli*

To evaluate expression of the FLAG-tagged truncated AHSV NS3 protein in different bacterial expression hosts, the recombinant plasmid pET41a-FNS3 was transformed into competent *E. coli* BL21(DE3), *E. coli* BL21-Star(DE3) and *E. coli* BL21-Gold(DE3) cells, respectively. The cells of IPTG-induced and control uninduced bacterial cultures were harvested, and the whole-cell lysates were analyzed by SDS-PAGE and Western blot analysis.

The modified truncated NS3 protein was expressed to very low levels in each of the *E. coli* strains and was barely detectable on a Coomassie-stained SDS-polyacrylamide gel (result not shown). Nevertheless, Western blot analysis of a duplicate unstained gel indicated that the anti-βgal-NS3 antiserum reacted with a protein of approximately 13 kDa that was present only in the whole-cell lysates of the induced recombinant *E. coli* BL21(DE3) and *E. coli* BL21-Star(DE3) bacterial cultures (Fig. 2.8, lanes 5 and 6, respectively). The size of this protein is in agreement with the expected size of the FLAG-tagged truncated NS3 protein, lacking the GST-tag moiety. In contrast, the antibody did not react with a similarly sized protein in the recombinant *E. coli* BL21-Gold(DE3) whole-cell lysate, indicating that the FLAG-tagged truncated NS3 protein is either not expressed in this *E. coli* host or is expressed at levels below the detection limit of the anti-βgal-NS3 antiserum (Fig. 2.8, lane 7).
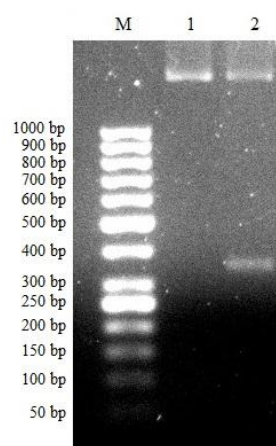
Figure 2.6: Agarose gel electrophoretic analysis of recombinant plasmid pGEM-FNS3, following digestion with *Eco*RI. Samples were loaded as follows: lane M – GeneRuler 50 bp DNA ladder (Thermo Scientific); lane 1 – *Eco*RI digest of non-recombinant pGEM-T Easy; lane 2 – *Eco*RI digest of recombinant pGEM-FNS3.
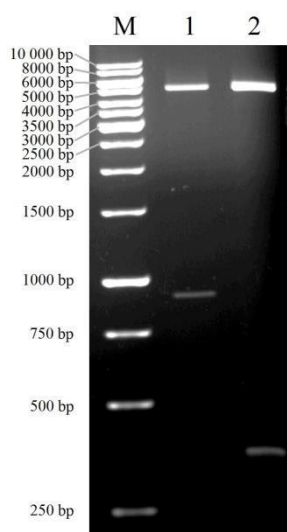
Figure 2.7: Agarose gel electrophoretic analysis of the recombinant pET41a-FNS3 bacterial expression plasmid, following digestion with both *Nde*I and *Xho*I. Samples were loaded as follows: lane M – GeneRuler 1 kb DNA ladder (Thermo Scientific); lane 1 – *Nde*I and *Xho*I digest of non-recombinant pET41a (+); lane 2 – *Nde*I and *Xho*I digest of recombinant pET41a-FNS3. Note should be taken that digestion of the non-recombinant pET41a (+) vector DNA with *Nde*I and *Xho*I yields two DNA fragments, which corresponds to the vector DNA and GST coding sequence, respectively.

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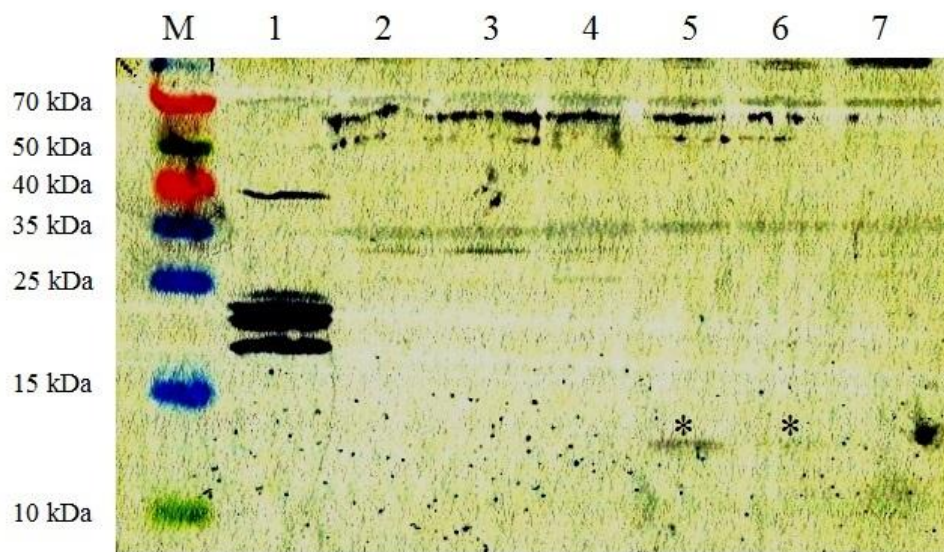
Figure 2.8: Western blot indicating expression of the FLAG-tagged truncated AHSV NS3 protein in recombinant *E. coli* BL21(DE3), *E. coli* BL21-Star(DE3) and *E. coli* BL21-Gold(DE3) strains. The Western blot was performed with whole-cell lysates of control uninduced (lanes 2-4) and IPTG-induced (lanes 5-7) bacterial cultures, and the modified truncated NS3 protein was detected with an anti-βgal-NS3 antiserum. The samples were loaded as follows: lane M – Spectra Broad Range Protein Ladder (Thermo Scientific); lane 1 – Positive control containing the full-length AHSV-3 NS3 protein expressed in the baculovirus-insect cell expression system; lane 2 – Whole-cell lysate prepared of an uninduced *E. coli* BL21(DE3) culture; lane 3 – Whole-cell lysate prepared of an uninduced *E. coli* BL21-Star(DE3) culture; lane 4 – Whole-cell lysate prepared of an uninduced *E. coli* BL21-Gold(DE3) culture; lane 5 – Whole-cell lysate prepared of an induced *E. coli* BL21(DE3) culture; lane 6 – Whole-cell lysate prepared of an induced *E. coli* BL21-Star(DE3) culture; lane 7 – Whole-cell lysate prepared of an induced *E. coli* BL21-Gold(DE3) culture. The expressed FLAG-tagged truncated NS3 protein is indicated by asterisks.

Based on the above results, indicating that the FLAG-tagged truncated NS3 protein was expressed to the highest level in *E. coli* BL21(DE3) cells, this expression host was used to express the modified truncated NS3 protein for use in subsequent pull-down experiments.

2.3.6   Pull-down experiments

To determine whether the AHSV NS3 protein is indeed capable of binding to the insect cell proteins SARA, Hsp70 and UB, pull-down experiments were performed. For this purpose, the GST fusion proteins immobilized on glutathione agarose beads were incubated with whole-cell lysates containing the expressed truncated NS3 protein to allow protein-protein interactions to occur. The beads were subsequently collected by centrifugation and the supernatant, containing unbound proteins, was retained. The beads, containing proteins from the whole-cell lysate bound to the GST fusion proteins, were washed to remove unbound and non-specifically bound proteins. As a control, a pull-down experiment was likewise performed using immobilized GST. The samples, as well as the supernatants were analyzed by Western blot analyses following SDS-PAGE.

If the truncated NS3 protein bound to the GST-tagged insect cell proteins, then it would be expected that the NS3 protein should be present in the glutathione agarose pellet samples. However, the results that were obtained indicated a lack of interaction between the truncated NS3 protein and the GST-SARA, GST-Hsp70 and GST-UB fusion proteins. This is based on the absence of an immunoreactive 13-kDa band corresponding with the expected size of the FLAG-tagged truncated NS3 protein (see Fig. 2.8, lane 5). Despite numerous attempts and the use of two different buffers, NP-40 (Fig. 2.9A) and TENT (Fig. 2.9B), no interaction between the proteins could be demonstrated.

2.4   DISCUSSION

Host cell proteins may play an important role in mediating virus release from infected cells. Several enveloped viruses recruit host proteins specifically to assist in the release of progeny virions from infected cells through budding (Calistri *et al.* 2009; Prange 2012). In contrast, relatively little is known about the contributions of host proteins to egress of non-enveloped viruses such as the orbiviruses. The NS3 protein of BTV has been shown to interact with the cellular proteins p11 and Tsg101, and these interactions were shown to indeed assist in the
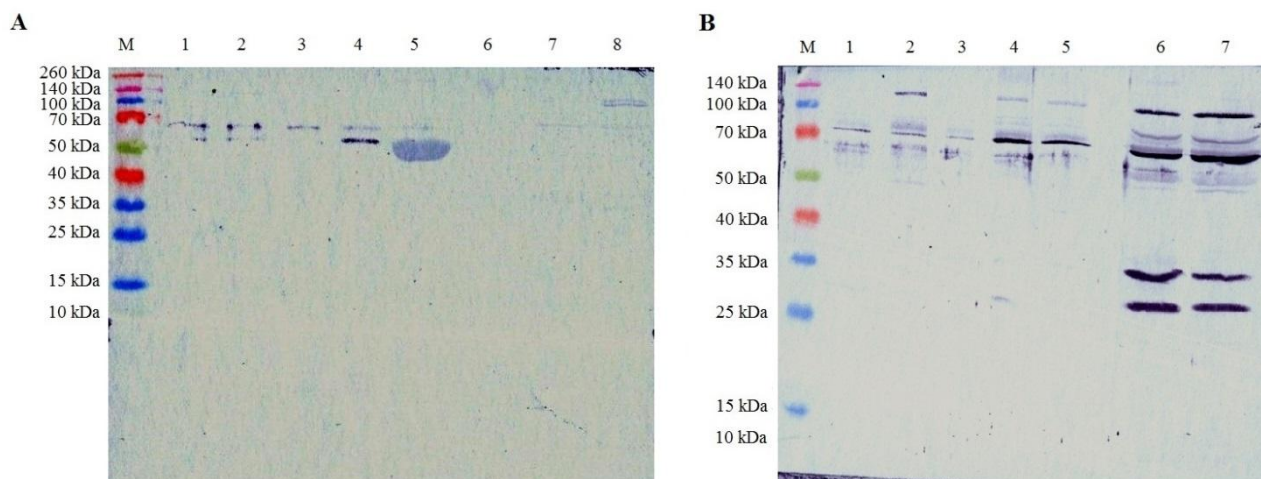
Figure 2.9: Western blot analyses of pull-down assays. The truncated NS3 protein of AHSV-3 was expressed in *E. coli* BL21(DE3) and whole-cell lysates were incubated with GST-SARA, GST-Hsp70 and GST-UB immobilized onto glutathione agarose beads. Two different buffers (NP-40 or TENT) were used during incubation and for washing of the protein complexes. Samples of the glutathione agarose pellets and whole-cell lysate supernatants were analyzed by Western blotting with anti-NS3 antisera. **(A)** Western blot showing the results of pull-down assays conducted in NP-40 buffer, and detection of the truncated NS3 protein with anti-NS3 antiserum. Samples were loaded as follows: lane M – Spectra Broad Range Protein Ladder (Thermo Scientific); lane 1 – supernatant from pull-down assay with GST-Hsp70; lane 2 – supernatant from pull-down assay with GST-UB; lane 3 – supernatant from pull-down assay with GST-SARA; lane 4 – supernant from pull-down assay with GST; lane 5 – glutathione agarose pellet from pull-down assay with GST-Hsp70; lane 6 – glutathione agarose pellet from pull-down assay with GST-UB; lane 7 – glutathione agarose pellet from pull-down assay with GST-SARA; lane 8 – glutathione agarose pellet from pull-down assay with GST. **(B)** Western blot showing the results of pull-down assays conducted in TENT buffer, and detection of the truncated NS3 protein with an anti-βgal-NS3 antiserum. The samples were loaded as follows: lane M – Spectra Broad Range Protein Ladder (Thermo Scientific); lane 1 – glutathione agarose pellet from pull-down assay with GST; lane 2 – glutathione agarose pellet from pull-down assay with GST-SARA; lane 3 – glutathione agarose pellet from pull-down assay with GST-Hsp70; lane 4 – glutathione agarose pellet from pull-down assay with GST-UB; lane 5 – supernatant from pull-down assay with GST-SARA; lane 6 – supernatant from pull-down assay with GST-Hsp70; lane 7 – supernatant from pull-down assay with GST-UB.

egress of virus particles from infected cells (Beaton *et al.* 2002; Wirblich *et al.* 2006; Celma and Roy 2009). In the case of AHSV, three insect cell proteins, SARA, Hsp70 and UB, were shown to interact with the NS3 protein in yeast two-hybrid screens (Beyleveld 2007; Barnes 2011). However, before undertaking investigations aimed at determining the biological relevance of these interactions, it is necessary to first confirm these NS3-host cell protein interactions with a second independent assay. To this end, the insect cell proteins were expressed in *E. coli* as GST fusion proteins and used with a truncated AHSV-3 NS3 protein in pull-down assays.

Over the past 15 years, the variety of hosts and vector systems for recombinant protein expression has increased dramatically. It is now possible to select from mammalian (Lundstrom 2003; Khan 2013), insect (Kost and Condreay 1999; Jarvis 2009) and prokaryotic (Schumann and Ferreira 2004; Schumann 2007) hosts and expression vector systems. Despite these developments, *E. coli* has remained one of the most attractive hosts for heterologous protein production, because of its ability to grow rapidly and at a high density on inexpensive substrates, its well characterized genetics, and the availability of a large number of expression vectors and mutant host strains (Baneyx 1999; Sørensen and Mortensen 2005; Samuelson 2011). Consequently, *E. coli* was selected as the expression host of choice in this study. To enable expression of the host cell peptides (SARA, Hsp70 and UB) and truncated AHSV NS3 protein in *E. coli*, the pET41a-c (+) family of expression vectors was selected for use. These expression vectors contain a tightly regulated bacteriophage T7 promoter, which directs the high-level cytoplasmic synthesis of heterologous proteins as fusions with a 220-amino-acid GST tag moiety. The GST tag may not only increase the solubility of fused peptides, but also facilitates purification of the recombinant fusion proteins from crude bacterial cell lysates by means of glutathione affinity chromatography under non-denaturing conditions (Studier *et al.* 1990).

The SARA, Hsp70 and UB gene fragments were each cloned into the pET41a-c (+) family of expression vectors. After induction with IPTG, protein samples of the derived recombinant *E. coli* strains were analyzed by SDS-PAGE. Recombinant proteins of the expected sizes were identified in the whole-cell lysates prepared from *E. coli* BL21(DE3) cultures containing the recombinant plasmids pET41c-SARA, pET41a-Hsp70 and pET41c-UB, respectively. The identity of the GST-SARA, GST-Hsp70 and GST-UB fusion proteins were subsequently confirmed by Western blot analysis using an anti-GST antibody. The GST fusion proteins

were expressed to high levels, but the GST-SARA fusion protein, in contrast to the GST-Hsp70 and GST-UB fusion proteins, was expressed as a predominantly insoluble protein.

The synthesis of target proteins as insoluble proteins and their subsequent accumulation into inclusion bodies is a frequent occurrence in *E. coli* (Villaverde and Carrio 2003; Sørensen and Mortensen 2005). The aggregated insoluble proteins are in general misfolded and thus biologically inactive (Thomas and Baneyx 1996; Villaverde and Carrio 2003). Under normal cellular conditions a subset of cytoplasmic proteins are able to fold spontaneously, while aggregation-prone proteins require the assistance of a number of molecular chaperones that interact reversibly with nascent polypeptide chains to prevent aggregation during the folding process (Hartl and Hayer-Hartl 2002). Aggregation of the GST-SARA fusion protein over-expressed in *E. coli* BL21(DE3) cells could therefore have resulted either from accumulation of high concentrations of folding intermediates or from inefficient processing by molecular chaperones. The precise physiochemical parameters that contribute to the formation of inclusion bodies remain unclear (Rudolph and Lilie 1996), but properties of the expressed target protein such as charge average, hydrophobicity and total number of residues may all aid in inclusion body formation (Wilkinson and Harrison 1991; Ventura 2005).

A variety of *in vitro* methods have been published describing refolding of insoluble proteins (Rudolph and Lilie 1996; Middelberg 2002; Burgess 2009), but this approach is often considered undesirable. The major obstacles are the poor recovery yields, the requirement for optimization of refolding conditions for each target protein and the strong possibility that the resolubilization process could affect the integrity of refolded proteins (Burgess 2009). Consequently, approaches to limit the *in vivo* aggregation of recombinant proteins are often preferred over protein denaturation-renaturation methods. Some commonly used approaches to reduce protein aggregation include growth of bacterial cultures at lower temperatures (Cabilly 1989; Schein 1993; Vasina and Baneyx 1996), alteration of the medium composition and pH (Sugimoto *et al.* 1991; Krause *et al.* 2010), and induction of inducible promoters with lower concentrations of the chemical inducer (Weickert *et al.* 1996; Donovan *et al.* 1996; Malakar and Venkatesh 2011). These approaches are all aimed at reducing the rate of protein synthesis so that the slower turnover of proteins may result in a greater yield of correctly folded soluble protein. In this study, an increased soluble protein fraction was obtained following expression of the GST-SARA fusion protein in the alternative expression host, *E. coli* BL21-Star(DE3). However, the yield of soluble GST-SARA was still much lower

compared to GST-Hsp70 and GST-UB (Fig. 2.5), thus necessitating that the above-mentioned approaches be explored in future studies as means to increase the yield of soluble GST-SARA.

To prepare the AHSV-3 NS3 protein for use with the GST fusion proteins in pull-down assays, a truncated NS3 gene, encoding amino acids 1 to 107 of the N-terminus, was cloned into the pET41a (+) expression vector. In this case, the cloning strategy entailed removal of the vector-borne GST coding sequence and the in-frame fusion of a FLAG epitope to the N-terminus of the truncated NS3 protein. SDS-PAGE analysis of whole-cell lysates indicated that the 13-kDa FLAG-tagged truncated NS3 protein was expressed successfully and its identity was subsequently confirmed by Western blot analysis using an anti-NS3 antiserum. The use of this truncated NS3 protein was preferred since reports have indicated that the full-length AHSV NS3 protein is expressed in an insoluble form in *E. coli* and is also cytotoxic to the expression host (Van Niekerk *et al.* 2001a; Meiring 2009). Furthermore, this truncated NS3 protein was used in previous studies to identify SARA, Hsp70 and UB as NS3-interacting proteins (Beyleveld 2007; Barnes 2011) and therefore contains the sites required for interaction with these proteins. In this study, the truncated NS3 protein was modified through the inclusion of a FLAG tag at its N-terminus. This was done with the aim of using co-immunoprecipitation as an alternative to pull-down assays to verify interaction of NS3 with the different host cell proteins. Specifically, it was envisaged to transfect a recombinant mammalian expression plasmid harbouring this modified truncated NS3 gene into BHK-21 cells. Following lysis of the transfected cells, the expressed tagged truncated NS3 protein could be immunoprecipitated with an anti-FLAG M1 monoclonal antibody, thereby allowing for co-immunoprecipation of proteins that interact directly with the NS3 protein. These protein complexes can be analyzed by SDS-PAGE and the co-immunoprecipitated proteins identified using mass spectrometry (Phizicky and Fields 1995; Miernyk and Thelen 2008). However, due to time constraints these experiments could not be performed in the current study.

In this study, pull-down assays were performed to verify protein-protein interactions by incubating the GST-SARA, GST-Hsp70 and GST-UB fusion proteins, immobilized on glutathione agarose beads, with a cell lysate containing the modified truncated NS3 protein. Unfortunately, no interaction between the viral and cellular proteins could be demonstrated (Fig. 2.9). Several reasons may explain these results. Firstly, despite using different volumes

of the NS3-containing whole-cell lysate, the concentrations of NS3 used in these assays may have been too low and thus precluded its detection in subsequent Western blot analyses. Secondly, it may be that the interactions between NS3 and the cellular proteins are transient in nature. Since pull-down assays are biased towards proteins that interact with high affinity and with slow kinetics of dissociation (Phizicky and Fields 1995; Berggard *et al.* 2007), it may therefore not be optimal for the detection of transient protein-protein interactions. Thirdly, despite using two buffers that differed with regard to their salt concentration (150 mM NaCl vs. 100 mM NaCl) and detergent (Triton X-100 vs. NP-40) (Wirblich *et al.* 2006), it may be that these buffers were too stringent and could therefore have reduced the interactions between NS3 and the respective host cell proteins, especially if these are low-affinity or transient interactions. Finally, by taking all of the above into consideration, it is possible that the pull-down assay is simply not sensitive enough and therefore necessitates the use of more sensitive methods to verify the protein-protein interactions.

To overcome the impediments highlighted above, several alternative approaches could be adopted in future studies. To address problems relating to the low expression level of the truncated NS3 protein, other expression systems should be investigated. A promising alternative to *E. coli* used in this study, is the baculovirus-insect cell expression system. This expression system has become one of the most powerful and versatile eukaryotic expression systems for the production of high levels of biologically active and functional recombinant proteins (Yin *et al*. 2007; Jarvis 2009), and the expression of foreign proteins may represent 50% of the total protein of an infected cell (Matsuura *et al.* 1987). Furthermore, the FLAG tag may be exploited to purify and thus concentrate the truncated NS3 protein. This could be achieved by, for example, immunoprecipitation with an anti-FLAG M1 monoclonal antibody, after which the NS3 protein can be eluted readily with EDTA (Hopp *et al.* 1988). The methodology can be extended to include radiolabelling of the newly synthesized truncated NS3 protein with [^{35}S]-methionine *in vivo*, thus allowing for sensitive detection and obviating the need for Western blot analyses.

More sensitive methods for detecting protein-protein interactions should also be considered. A promising technique is fluorescence resonance energy transfer (FRET) analysis. In this approach, the bait and prey proteins are fused to two different fluorescent tags with overlapping emission/excitation spectra. If both the proteins are in close proximity to each other, excitation of the first fluorophore (donor) leads to energy transfer to the second

fluorophore (acceptor). The acceptor fluorescence can subsequently be observed either *in vitro* with a fluorometer or in cells by confocal microscopy (Masi *et al.* 2010; Gau *et al.* 2011). Alternatively, surface plasmon resonance (SPR) studies can be undertaken. In this case, the bait protein immobilized on the surface of a sensor chip is probed by injection of prey protein onto the surface, and protein interaction is detected online via a change in refractive index at the sensor surface. The protein can then be eluted and identified by mass spectroscopy (Rich and Myszka 2001; Huber and Mueller 2006). The main advantages associated with SPR analysis are that very small amounts of sample are needed (mg or sub-mg may suffice), no labelling is required and the method can provide information on the rates of association and dissociation of the interacting proteins (Berggard *et al.* 2007). Albeit not conclusive evidence for interaction, *in vivo* co-localization of the bait and prey proteins in a cell may be studied by confocal microscopy (Miyashita 2004). This approach, however, requires the availability of highly specific primary antibodies raised against the proteins of interest. After fixing of the cells and incubation with primary antibodies, the cells are incubated with two fluorophore-labelled secondary antibodies and detected. By taking advantage of the fact that the two fluorophores display different emission maxima, the intracellular localization of the proteins can be monitored and, if the two proteins are co-localized, the fluorescent probes will also be co-localized.

In conclusion, despite being able to express the bait (GST-SARA, GST-Hsp70 and GST-UB) and prey (FLAG-tagged truncated NS3) proteins in *E. coli*, no interaction between NS3 and the respective host cell proteins could be demonstrated in pull-down assays. However, a number of factors that may have potentially influenced the results were identified. Thus, further experiments, by taking the above suggestions into account, are required before a definitive conclusion can be drawn as to whether or not the NS3 protein of AHSV interacts with the SARA, Hsp70 or UB proteins.

CHAPTER 3

RNAi-MEDIATED KNOCKDOWN OF SARA, HSP70 AND UBIQUITIN GENE EXPRESSION, AND ITS EFFECT ON AHSV EGRESS

3.1   INTRODUCTION

The characterization of African horsesickness virus (AHSV) NS3 function has been confined to the study of NS3 expressed in bacterial and eukaryotic expression systems (Van Staden *et al.* 1995; Stoltz *et al.* 1996; Van Niekerk *et al.* 2001a), and more recently by studying reassortant viruses between different AHSV serotypes (Meiring *et al.* 2009). These investigations have suggested a role for NS3 in pathogenesis, membrane permeability and virus release. In addition to lytic virus release, it has been observed by immune-electron microscopy that AHSV can leave infected mammalian cells by budding at the plasma membrane prior to cell lysis (Stoltz *et al.* 1996). Studies on the NS3 protein of the related bluetongue virus (BTV) have indicated that the protein recruits different cellular proteins to facilitate NS3-mediated non-lytic release of virions (Beaton *et al.* 2002; Wirblich *et al.* 2006; Celma and Roy, 2011).

Although the AHSV NS3 protein was shown previously to interact with different insect cell proteins such as SARA, heat shock protein 70 (Hsp70) and ubiquitin (UB) in yeast two-hybrid screens (Beyleveld 2007; Barnes 2011), the significance of these interactions have not yet been investigated. In the previous Chapter, attempts were made to confirm these interactions *in vitro* by making use of pull-down assays. Although interaction between NS3 and the different insect cell proteins could not be demonstrated using this approach, it can nevertheless be envisaged that a clearer understanding of the possible role of these cellular proteins in AHSV egress may be obtained by observing phenotypic consequences resulting from their inactivation. In this regard, RNA interference (RNAi) can provide a useful investigative tool that may greatly facilitate studies aimed at generating such loss-of-function phenotypes (Paddison 2008; Belles 2010; Pache *et al.* 2011).

RNA interference (RNAi) is a conserved gene silencing mechanism that recognizes dsRNA as a signal to trigger the sequence-specific degradation of homologous mRNA (Fire *et al.* 1998; Montgomery *et al.* 1998; Tuschl *et al.* 1999). Biochemical and genetic studies in several experimental systems have indicated that dsRNA-induced gene silencing proceeds via a multi-step mechanism. Initially, long dsRNA molecules are recognized and cleaved into 21- to 23-nt small interfering RNAs (siRNAs) by the action of Dicer, a dsRNA-specific endonuclease that is a member of the RNase III family (Bernstein *et al.* 2001). The siRNA is unwound and individual strands are incorporated into a protein complex known as an RNA-

induced silencing complex (RISC). The activated RISC, *i.e.* RISC containing the antisense RNA or guide strand, is responsible for cleaving mRNA that is complementary to the guide strand in the complex (Agrawal *et al.* 2003; Ameres *et al.* 2007; Wilson and Doudna 2013). This, in turn, silences expression of the corresponding protein due to the reduction in the pool of mRNA available for translation (Fig. 3.1).
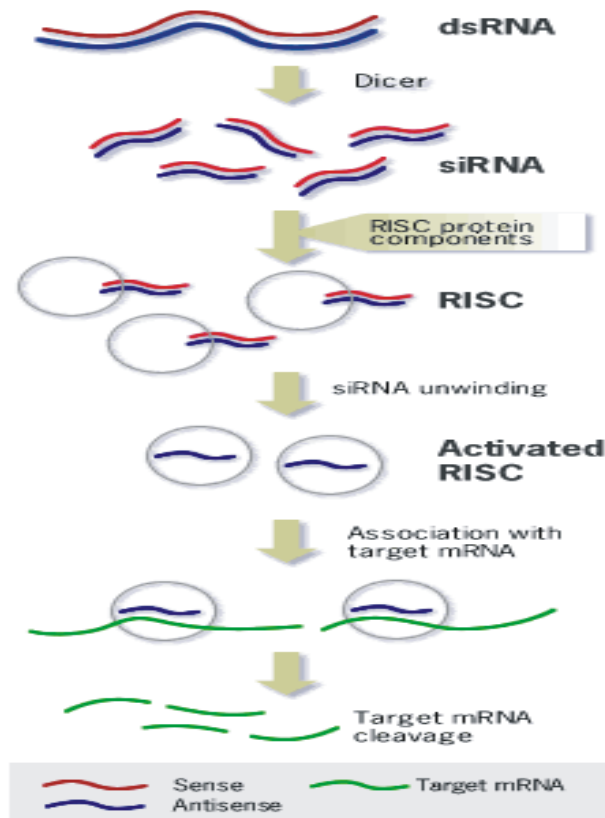
Figure 3.1: Schematic diagram of RNAi-mediated gene silencing in mammals. The double-stranded RNAs are processed by Dicer to produce siRNAs. The siRNAs become associated with the RISC complex, after which the antisense strand guides the complex towards the cognate mRNA, resulting in endonucleolytic cleavage of the mRNA (Whitehead *et al.* 2009).

Despite its utility in diverse biological systems, the application of RNAi to mammalian cells was initially restricted since the introduction of dsRNA longer than 30 bp triggered an interferon (IFN) response, which results in non-specific gene silencing due to an overall shutdown of protein synthesis (Williams 1997; Stark *et al.* 1998). Predominant amongst the responses triggered is activation of protein kinase R (PKR), a kinase that is activated by dimerization in the presence of dsRNA. Activated PKR subsequently phosphorylates the

eukaryotic translation initiation factor eIF2α, causing a non-specific translational shutdown (Manche *et al.* 1992). In addition, dsRNA also activates 2',5'-oligoadenylate synthetase, the product of which is an essential co-factor for a non-specific ribonuclease, RNase L, resulting in the degradation of cellular RNA (Minks *et al.* 1979). These problems, however, can be overcome by introducing synthetic 21- to 23-nt siRNA duplexes directly into the cytoplasm of mammalian cells (Elbashir *et al.* 2001; Caplen *et al.* 2001; Whitehead *et al.* 2009). The synthetic siRNAs avoid provoking the PKR response by virtue of their small size and are incorporated directly into the RNAi pathway by mimicking the products of the Dicer enzyme. For monitoring and validation of the silencing effect, standard molecular biology techniques are typically used. Methods for quantification of mRNA levels include Northern blot hybridization and quantitative reverse transcriptase (RT)-PCR or real-time PCR, whereas immunodetection methods, such as Western blot analysis and immunoprecipitation with protein-specific antibodies, are used to quantify the level of protein (Sandy *et al.* 2005).

To determine whether the SARA, Hsp70 and UB proteins may have biological relevance in the non-lytic release of AHSV from infected cells, the primary aims of this part of the study were to silence expression of the cellular proteins using appropriate siRNAs and then to determine the effect of gene silencing on AHSV release from mammalian cells.

3.2   MATERIALS AND METHODS

3.2.1   Mammalian cell culture and virus

Baby hamster kidney-21 (BHK-21; ATCC CL-10) cells were propagated as monolayers in 75-cm^2 tissue culture flasks. The cells were cultured in Eagle's Minimal Essential Medium (MEM) with Earle's Balanced Salt Solution (EBSS) and L-glutamine (HyClone), supplemented with 5% (v/v) foetal bovine serum (FBS), 1% (v/v) non-essential amino acids (NEAA) and antibiotics (10 000 U/ml of penicillin, 10 000 µg/ml of streptomycin and 25 µg/ml of amphotericin B) (HyClone). The flasks were incubated at 37°C in a humidified incubator with a constant supply of 5% CO_2. AHSV-3 was kindly provided by Mr F. Wege (Department of Genetics, University of Pretoria) and used for all cell infections.

3.2.2   Small interfering RNAs (siRNAs)

The lack of SARA, Hsp70 and UB gene sequences specific to the hamster genome precluded the design of siRNAs and therefore siRNAs were obtained from commercial sources. siRNAs against mouse Hsp70 (mHsp70) and mouse UB (mUB) mRNA were obtained from Qiagen, whereas a siRNA against mouse SARA (mSARA) mRNA was obtained from Santa Cruz Biotechnology. A negative (non-silencing) control siRNA, designated UN-siRNA, was purchased from Qiagen and reportedly lacks homology to known viral and cellular genes. All siRNAs were supplied as lyophilized, desalted duplexes and were suspended in the supplied RNase-free water at a concentration of 10 µM (mSARA-siRNA) and 20 µM (mHsp70-siRNA, mUB-siRNA and UN-siRNA) prior to storage at -20°C. The siRNAs used in this study, with the exception of the mSARA-siRNA, are shown in Table 3.1. With regards to the latter siRNA, the target sequence is not known, as the only information provided by the commercial supplier was that it consists of a pool of three target-specific 20- to 25-nt siRNAs designed to knockdown mouse SARA gene expression.

Table 3.1: siRNAs used in this part of the study

siRNA	siRNA sequences	Target mRNA
mHsp70-siRNA	**Sense:** 5'-GGUCGUUCUUCCCGGAGGAdTdT-3' **Antisense:** 5'-UCCUCCGGGAAGAACGACCdGdG-3'	Hsp70
mUB-siRNA	**Sense:** 5'-AAGUUUCAAUAAUAGCUGAdTdT-3' **Antisense:** 5'-UCAGCUAUUAUUGAAACUUdGdT-3'	Ubiquitin
UN-siRNA	**Sense:** 5'-UUCUCCGAACGUGUCACGUdTdT-3' **Antisense:** 5'-ACGUGACACGUUCGGAGAAdTdT-3'	No homology

3.2.3   Transfection of BHK-21 cells with siRNA

BHK-21 cells were seeded in 24-well tissue culture plates to reach 60% confluency within 24 h of incubation at 37°C in a CO_2 incubator. The BHK-21 cells were then transfected with the respective siRNAs using Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's instructions. For each transfection, 1 µl of Lipofectamine 2000 reagent was diluted in 50 µl of incomplete MEM medium (serum- and antibiotic-free). Likewise, 10 pmol of the relevant siRNA was also diluted in 50 µl of incomplete MEM medium. Following incubation at room temperature for 5 min, the two solutions were mixed and incubated at

room temperature for 20 min to allow the formation of RNA-lipofectamine complexes. The cell monolayers were prepared for transfection by rinsing three times with 1 ml of incomplete MEM medium, followed by addition of 500 µl of antibiotic-free MEM medium containing 5% (v/v) FBS. The BHK-21 cell monolayers were subsequently overlaid with the RNA-lipofectamine complexes and the tissue culture plates were incubated at 37°C for 12 h in a CO_2 incubator. Following incubation, the cell monolayers were transfected for a second time, as described above, and incubated for a further 12 h in the CO_2 incubator. Following incubation, the cell monolayers were processed for RNA isolation as described below.

3.2.4   Real-time quantitative PCR

3.2.4.1   Primers

As the experiments were performed in mammalian BHK-21 cells, primers were designed to target the mammalian homologues of the insect genes SARA, Hsp70 and UB. Considering the lack of hamster genomic sequences available in the National Center for Biotechnology Information (NCBI) database, the deduced amino acid sequences of the insect gene segments were thus used to identify highly similar mammalian proteins with BLASTP (Altschul *et al*. 1990). The mammalian SARA, Hsp70 and UB homologue genes were then respectively aligned using BioEdit v.7.0.9.0 (Hall 1999) and conserved sequences were used for the design of gene-specific primer pairs. The SARA primer pair was designed by aligning the SARA mRNA sequences of *Mus musculus* (accession number: NM_183300) and *Rattus norvegicus* (accession number: NM_001107952), the Hsp70 primer pair was designed by aligning the *M. musculus* heat shock protein 1A mRNA (accession number: NM_010479) and *R. norvegicus* heat shock 70 kDa protein 1A mRNA (accession number: NM_031971), and the UB gene primer pair was designed based on the alignment of *M. musculus* ubiquitin B (accession number: NM_011664) and *R. norvegicus* ubiquitin B (accession number: NM_138895). The primers, indicated in Table 3.2, were designed using PerlPrimer v.1.1.20 (Marshall 2004) in such a way that the amplification product was between 129 and 161 bp in size. In addition to the above, primers targeting the β2-microglobulin (β2M) housekeeping gene was provided by Dr. L. Stassen (Department of Microbiology and Plant Pathology, University of Pretoria).

Table 3.2: Primers used in real-time quantitative PCR

Target mRNA	Primer name	Primer sequence	Amplicon	T_m
SARA	RT-SARA Fwd	5'-GACTCCGTGTGACGCTTGACTCAG-3'	129 bp	56ºC
	RT-SARA Rev	5'-GGCAGGCTCCTCCATGAATGAC-3'		
Hsp70	RT-Hsp70 Fwd	5'-GGAGTTCGTGCACAAGCGG-3'	161 bp	57ºC
	RT-Hsp70 Rev	5'-GCCTCTAATCCACCTCCTCGAT-3'		
UB	RT-UB Fwd	5'-GCACCTGGTCCTCCGTCT-3'	138 bp	53ºC
	RT-UB Rev	5'-GGGATGCCCTCTTTATCCTG-3'		
β2M	RT-β2M Fwd	5'-AGTGGAGCTGTCAGATCTGTCCTTC-3'	138 bp	61ºC
	RT-β2M Rev	5'-TGACCACCTTGGGCTCCTTC-3'		

3.2.4.2 RNA extraction

Total RNA was extracted with the Nucleospin RNA II Total RNA Isolation kit (Macherey-Nagel) according to the manufacturer's instructions. The culture medium was aspirated and the cells were rinsed once with 1 ml of 1 × PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na$_2$HPO$_4$.2H$_2$O, 1.4 mM KH$_2$PO$_4$; pH 7.4). Subsequently, 350 µl of the supplied lysis buffer RA1 and 3.5 µl of β-mercaptoethanol were pipetted directly onto the cell monolayer. The viscosity of the cell lysate was first reduced by centrifugation through a NucleoSpin® filter at 12 000 rpm for 1 min, after which 350 µl of freshly prepared 70% ethanol was added to the flow-through and pipetted vigorously (at least 5 times). Following centrifugation of the cell lysate through a NucleoSpin® RNA II column at 12 000 rpm for 30 s, 350 µl of Membrane Desalting buffer was added to the column and centrifuged at 12 000 rpm for 1 min. To remove contaminating DNA, the column was treated at room temperature for 15 min with the supplied RNase-free DNase I. Following incubation, the column was washed once with 250 µl of buffer RA2 and three times with 250 µl of buffer RA3 (12 000 rpm for 30 s). The RNA was then eluted from the column in 60 µl of RNase-free water. The RNA samples were analyzed on a 1% agarose gel (Section 2.2.4) and stored at -70°C until required.

3.2.4.3 cDNA synthesis

The extracted RNA was reverse transcribed with the RevertAid H Minus First Strand cDNA Synthesis kit (Thermo Scientific) according to the manufacturer's instructions. For reverse transcription, 5 µg of the RNA sample was mixed with 1 µl of random hexamer primers and nuclease-free water was added to a final volume of 12 µl. Following incubation at 65°C for 5

min, 4 µl of 5 × Reaction buffer, 1 µl of RiboLock RNase inhibitor (20 U/µl), 2 µl of a 10 mM dNTP mixture and 1 µl of RevertAid H Minus M-MuLV reverse transcriptase (200 U/µl) was added to the reaction. The reaction mixture was incubated at 42°C for 1 h and the enzyme was then inactivated by incubation at 70°C for 5 min.

3.2.4.4 Control PCR reactions

To confirm the absence of contaminating DNA in the RNA preparations and to determine the amplification specificity of the designed primers (Table 3.2), conventional PCR reactions were performed. Each of the PCR reaction mixtures contained 1.5 µl of the RNA or cDNA preparation, 10 pmol of each the gene-specific forward and reverse primer, 200 µM of each dNTP, 1 × Reaction buffer, 1.5 mM $MgCl_2$, 1 U of SuperTherm *Taq* DNA polymerase (Seperation Scientific) and nuclease-free water to a final volume of 25 µl. Reactions mixtures lacking template were also included as controls. Thermal cycling was performed in a Eppendorf Mastercycler® Gradient thermal cycler with the following cycling parameters: initial denaturation for 3 min at 94ºC, followed by 30 cycles of denaturation for 15 s at 94ºC, primer annealing for 15 s at the temperatures indicated in Table 3.2 for the respective primer pairs, and extension for 15 s at 72ºC. After a final extension for 1 min at 72ºC, the reaction mixtures were analyzed by electrophoresis on a 2% (w/v) agarose gel in the presence of an appropriate DNA molecular weight marker.

3.2.4.5 Real-time quantitative PCR (RT-qPCR)

Real-time quantitative PCR was performed using the KAPA SYBR® FAST qPCR kit Master Mix (2×) Universal (KAPA Biosystems) and the LightCycler 1.5 instrument (Roche). Each reaction mixture, prepared in glass capillaries, contained 1.5 µl of undiluted cDNA, 2.5 µl of the supplied BSA, 2 pmol of gene-specific forward and reverse primers (Table 3.2), 10 µl of 2 × KAPA Master Mix and nuclease-free water to a final volume of 20 µl. The reaction mixtures were incubated for 3 min at 95°C to activate the KAPA SYBR® DNA polymerase and then subjected to thermal cycling. In the case of reactions targeting the SARA and Hsp70 genes, the reaction mixtures were subjected to 40 cycles of denaturation for 3 s at 95°C, primer annealing for 20 s at 56°C (primers RT-SARA Fwd and Rev) or 57°C (primers RT-Hsp70 Fwd and Rev) and extension at 72°C for 1 s. For reactions targeting the ubiquitin (UB) and β2M genes, identical thermocycling profiles as above were used, but with the following modifications. In the case of reactions targeting the UB gene the primer annealing

temperature was 53°C and the number of cycles was reduced to 30, whereas the primer annealing temperature of reactions targeting the β2M housekeeping gene was 61°C and the number of cycles was 35. For each target gene, reaction mixtures identical to that described above were prepared, except template was omitted. To confirm specific amplification, melt-curve analysis of the amplicons was performed with the LightCycler v.4.1.1.21 software and an aliquot of each reaction mixture was also analyzed on a 2% (w/v) agarose gel in the presence of an appropriate DNA molecular weight marker.

3.2.4.6   Data analysis

For Crossing Point (C*p*) determination, the Second Derivate Maximum Method was used, which is included in the LightCycler v.4.1.1.21 software (Roche). This data was subsequently used to compute the relative expression of the target gene (SARA, Hsp70 or UB) normalized to an endogenous reference gene (β2M) with the Relative Expression Software Tool 2009 (REST© 2009) (Pfaffl *et al*. 2002). Group-wise comparisons and statistical analyses of the relative expression results were performed using the same software program.

3.2.5   Transfection of BHK-21 cells with siRNA and infection with AHSV-3

BHK-21 cells were seeded in 24-well tissue culture plates to reach 60% confluency within 24 h of incubation at 37°C in a CO_2 incubator. The BHK-21 cell monolayers were prepared for transfection and transfected twice with the siRNAs using Lipofectamine 2000 reagent (Invitrogen), as described above (Section 3.2.3). The transfected cells were then infected with AHSV-3. For infections, the BHK-21 cell monolayers were rinsed once with incomplete MEM medium and then infected with AHSV-3 at a multiplicity of infection (MOI) of 1 plaque forming unit (pfu)/cell. Virus infections were performed at 37°C for 1 h, after which the inoculum was aspirated and 1 ml of complete MEM medium was added to the cell monolayer. The tissue culture plates were incubated at 37°C in a CO_2 incubator. At 24 h post-infection, the cell monolayers were processed for virus titrations.

3.2.6   Virus titration

Extracellular virus titres were determined by collecting the supernatant of siRNA-treated, virus-infected cells. The samples were centrifuged at 3000 rpm for 5 min and the supernatant was transferred to a clean, sterile 1.5-ml Eppendorf tube for use in subsequent titrations. For determination of intracellular (cell-associated) virus titres, cells were removed from the wells

by addition of 1 ml of 2 mM Tris (pH 8) and added to the corresponding cell pellet previously collected. The cells were lysed by passing the cells 10 times through a 25G hypodermic needle. To determine the viral titres, plaque assays were performed on BSR cells, a clone of BHK-21 cells, according to the method described by Oellerman (1970) with the following modifications. Confluent BSR cell monolayers in a 6-well tissue culture plate were infected with serial dilutions (10^{-3}-10^{-5}) of virus in 2 mM Tris (pH 8). Following incubation at 37ºC for 1 h, the inoculums were aspirated and the cells overlaid with 2 ml of a 0.7% agarose solution (SeaKem® LE agarose) prepared in complete MEM medium. The tissue culture plates were incubated at 37ºC for 72 h in a CO_2 incubator and the cell monolayers were then stained with 0.1% (w/v) MTT [(3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide prepared in $1 \times$ PBS]. Following incubation at 37ºC for 24 h, the plaques were counted. Total virus yield was calculated as the sum of released and cell-associated virus, and virus release was expressed as the percentage released virus versus total virus (Meiring *et al.* 2009).

3.2.7   Statistical analysis

Experimental data from virus titrations is presented graphically by plotting means and reporting the standard deviation (SD) of the mean via error bars. The statistical significance of differences in two-sample-comparisons was calculated using GraphPad Prism v5.03 GraphPad Software, Inc.) statistical software by making use of a unpaired Student's *t*-test.

3.3   RESULTS

3.3.1   Knockdown of SARA, Hsp70 and UB gene expression in BHK-21 cells

RNAi functions by silencing target gene expression at a post-transcriptional level and has been used successfully in previous studies to determine the importance of the cellular protein Tsg101 in NS3-mediated non-lytic virus release (Wirblich *et al.* 2006). Towards determining whether the cellular proteins SARA, Hsp70 and UB may likewise play a role in facilitating NS3-mediated non-lytic virus release, it was necessary to determine whether the genes encoding these respective proteins could be knocked down with an RNAi approach. For this purpose, chemically synthesized siRNAs directed against the SARA, Hsp70 and UB mRNA were obtained from commercial sources and designated mSARA-siRNA, mHsp70-siRNA and mUB-siRNA, respectively.

To determine whether the above siRNAs could knockdown mRNA synthesis of their respective target genes, BHK-21 cell monolayers were transfected twice with the respective siRNAs or a control non-silencing siRNA (UN-siRNA). At 12 h post-second transfection, total RNA was isolated from the cells and subjected to reverse transcription followed by real-time quantitative PCR. In this study, β2M was used as an endogenous reference gene for data normalization and the extent of knockdown in siRNA-treated samples compared with those of the control UN-siRNA treated cells was determined with REST$^{©}$ 2009 software. This software program calculates changes in the transcription level of the gene of interest and the factor by which transcription is either up-regulated or down-regulated (Pfaffl *et al*. 2002).

The results, presented in Fig. 3.2 as Whisker-box plots, indicated that in BHK-21 cells transfected with either mSARA-siRNA or mUB-siRNA, transcription of the SARA and UB genes were reduced by a mean factor of 0.372 (63%) and 0.441 (56%), respectively, as compared with cells transfected with the control non-silencing siRNA (UN-siRNA). Analysis of the data indicated that the difference between the SARA and UB transcripts in cells transfected with the respective siRNAs and that of cells transfected with the control UN-siRNA was statistically significant ($p < 0.1$). In contrast, Hsp70 mRNA synthesis was reduced to a much lower extent. Transfection of BHK-21 cells with mHsp70-siRNA down-regulated transcription of the Hsp70 gene by a mean factor of 0.969 (3%) and was statistically insignificant compared with cells transfected with the control UN-siRNA ($p > 0.1$).

The possibility of DNA contamination in the RNA preparations used above was eliminated by performing RNase-free DNase I treatments and verified by subjecting the samples to PCR amplification, using *Taq* DNA polymerase and the gene-specific primer pairs (Table 3.2). No amplicons were obtained from control reaction mixtures lacking template or from the RNA preparations that were subsequently used for cDNA synthesis. In contrast, amplicons of the expected sizes were obtained when cDNA was used as template DNA in the above PCR reactions (129 bp for SARA; 161 bp for Hsp70; 138 bp for UB; 138 bp for β2M), thus confirming that the designed primer pairs were capable of amplifying the intended target genes of BHK-21 cells (Fig. 3.3). Furthermore, the amplification specificity of the real-time quantitative PCR was verified by agarose gel electrophoresis and a single amplicon of the expected length for each target was obtained (results not shown).
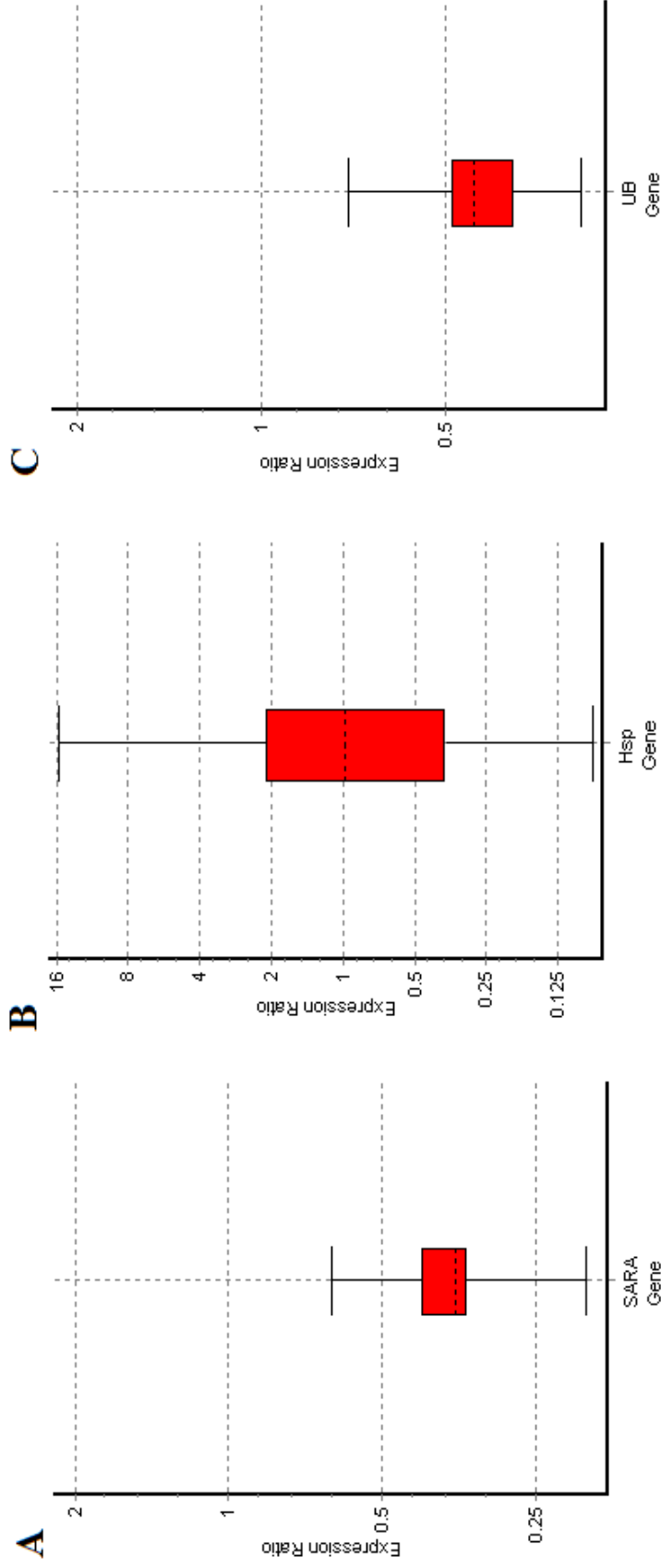
Figure 3.2: Knockdown of SARA, Hsp70 and UB gene expression in BHK-21 cells. At 24 h post-transfection the ratio of target gene mRNA transcripts in cells transfected with mSARA-siRNA, mHsp70-siRNA and mUB-siRNA, respectively, to that in cells transfected with a control non-silencing siRNA (UN-siRNA) was determined by real-time quantitative PCR using the β2M gene as endogenous control. For each Whisker-box plot, boxes represent the interquartile range and the dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations. Data are shown as the means ± SD of three independent experiments.

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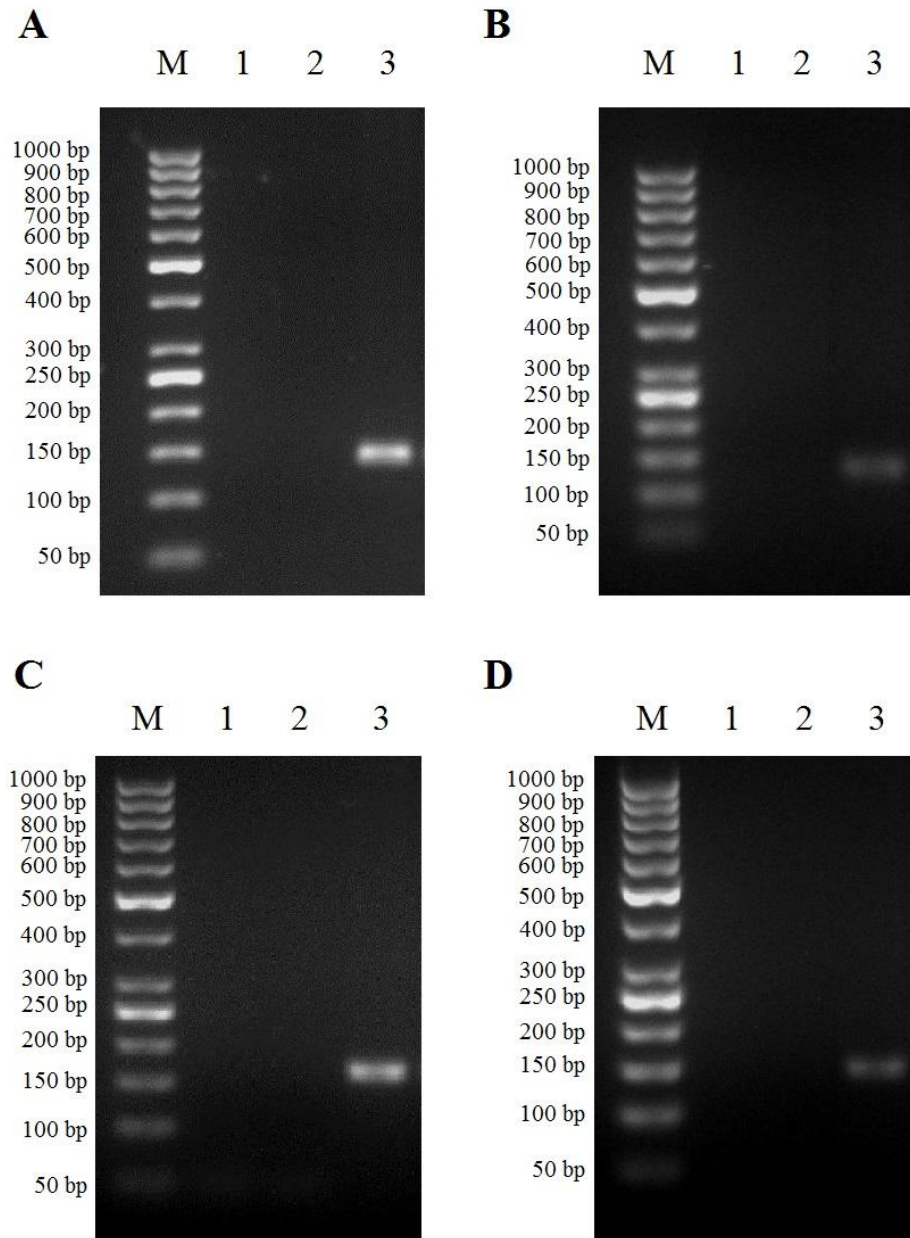
Figure 3.3: Agarose gel electrophoretic analysis of the amplification products generated by conventional PCR. The PCR reactions were performed using either total RNA or the synthesized cDNA as template, together with the β2M **(A)**, SARA **(B)**, Hsp70 **(C)** or UB **(D)** gene-specific primer pairs. The samples were loaded as follows: lane M - GeneRuler 50 bp DNA ladder (Thermo Scientific); lanes 1 – no-template negative PCR control; lanes 2 – PCR with RNA as template; lanes 3 – PCR with cDNA as template.

Based on the above, it was concluded that only the SARA- and UB-directed siRNAs were capable of reducing the accumulation of SARA and UB mRNA in BHK-21 cells. Although expression of these cellular genes in the siRNA-treated cells was reduced by 63% and 56%, respectively, this is below the level of knockdown (at least 70%) generally accepted as being meaningful in loss-of-gene function studies (Caplen *et al.* 2001; Banan and Puri 2004; Hsieh et *al.* 2004). Nevertheless, since a reduction in the respective transcript levels was obtained, this approach was pursued to determine its effect on AHSV egress from infected cells.

3.3.2   Effect of SARA and UB knockdown on virus release

The effect of knocking down SARA and UB gene expression on virus release was subsequently investigated. For this purpose, BHK-21 cell monolayers were transfected twice with the relevant siRNAs and then infected with AHSV-3, after which the titres of infectious virus present both intracellularly (cell-associated) and in the culture medium (extracellular) was determined. The respective titres, as determined at 24 h post-infection, were combined to represent the total virus yield, whereas virus release was calculated by expressing the titres obtained from the culture medium as a percentage of the total titre. Despite not being able to knockdown Hsp70 gene expression with the available mHsp70-siRNA, BHK-21 cells transfected with this siRNA prior to virus infection were nevertheless included as an internal control for these experiments.

BHK-21 cells that were treated with the control non-silencing siRNA (UN-siRNA) or with the SARA- and UB-directed siRNAs prior to infection with AHSV-3 all produced similar levels of total progeny virus, ranging between 4.0-5.0 $\times$ 10^6 pfu/ml (Fig. 3.4A). Not only do these results indicate that virus replication occurred in the cells during the 24-h incubation period, but also that transfection of the cells with the SARA- and UB-directed siRNAs did not non-specifically interfere with virus replication as compared to cells transfected with the control non-silencing siRNA. The viruses were found to be predominantly cell-associated, and the lowest percentage (30%) of AHSV-3 virions was released from the virus-infected cells that had been transfected with mUB-siRNA (Fig. 3.4B). In contrast, 35% of the AHSV-3 virions were released from BHK-21 cells that had been treated with the mSARA-siRNA. The levels of virus release from these siRNA-treated BHK-21 cells were similar to that of cells treated with the control non-silencing UN-siRNA (32%). As expected, results obtained for BHK-21 cells transfected with the mHsp70-siRNA yielded similar results to those

A

B
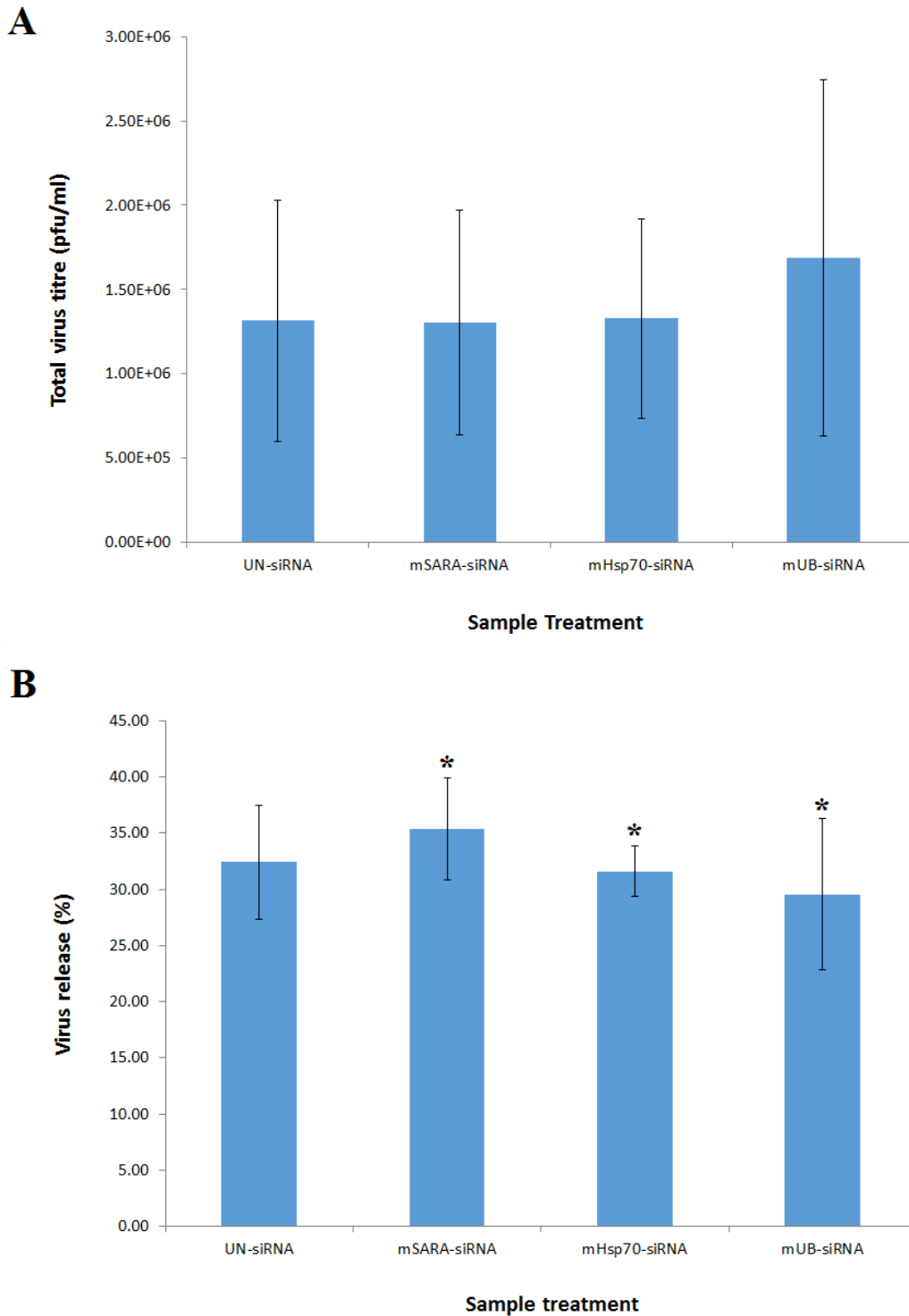
Figure 3.4: Effect of knocking down SARA, UB and Hsp70 gene expression on the total infectious virus yield (**A**) and the percentage virus released (**B**) of AHSV-3 in BHK-21 cells. The cells were transfected with the indicated siRNAs and control UN-siRNA prior to infection with AHSV-3. At 24 post-infection, the cell-associated and released virus was titrated. Values indicate the means ± SD of three independent experiments. Values were compared to that obtained for control non-silencing UN-siRNA treated cells by the Student's *t*-test, where * indicates no statistically significant difference ($p > 0.05$).

obtained for cells transfected with the UN-siRNA. Not only were the total titres near-identical (3.94 and 3.98 $\times$ 10^6 pfu/ml), but also the percentage of released virus (32 and 31%). These results therefore serve to validate the results obtained above for BHK-21 cells treated with mSARA-siRNA and mUB-siRNA.

Based on the above results, indicating that suppressing SARA and UB gene expression does not reduce AHSV egress, it appears that that these proteins may not play a role in mediating non-lytic virus release from the infected BHK-21 cells.

3.4   DISCUSSION

The orbivirus non-structural protein NS3 plays a key role in virus release and the mechanisms underlying this process is an active area of research (Stoltz *et al.* 1996; Beaton *et al.* 2002; Han and Harty 2004; Wirblich *et al.* 2006; Meiring *et al.* 2009). Studies have revealed that different host cell proteins, in conjunction with NS3, may facilitate the non-lytic release of BTV from infected cells (Celma and Roy 2009; Celma and Roy 2011). Although the insect cell proteins SARA, Hsp70 and UB were shown to interact with the NS3 protein of AHSV in yeast two-hybrid screens (Beyleveld 2007; Barnes 2011), no interaction between the respective proteins could be demonstrated by pull-down assays (Chapter 2). Therefore, an RNAi-based approach was adopted to investigate the involvement of these host cell proteins in AHSV release. It was reasoned that if SARA, Hsp70 or UB does indeed play a role in virus release, either directly or indirectly through binding to NS3, then knocking down their expression should interfere with the process of virus release, leading to a reduction in the amount of released virion particles.

In this study, pre-designed and validated siRNAs capable of silencing mouse SARA, Hsp70 and UB gene expression were obtained from commercial sources, as a lack of sequence information precluded the design of siRNAs specifically targeting the homologous genes of BHK-21 cells. Since the hallmark of RNAi is the degradation of targeted mRNA transcripts, the efficiency of the respective siRNAs to down-regulate expression of their target genes in BHK-21 cells was investigated using real-time quantitative PCR. Analysis of the results indicated that mSARA-siRNA and mUB-siRNA reduced target gene expression by 63% and 56%, respectively, whereas mHsp70-siRNA was most inefficient (3% reduction). Notably,

the same results were obtained in three independent experiments, as well as in experiments in which three different concentrations of the siRNAs were used (10, 20 and 40 pmol; results not shown). These results, and especially the low efficacy of mHsp70-siRNA, are therefore not attributable to differences in transfection efficiencies.

Despite RNAi having become a powerful and widely used approach for the analysis of gene function (Elbashir *et al.* 2002; Prudencio and Lehmann 2009; Belles 2010; Houzet and Jeang 2011), there are several obstacles to the use of this approach in mammalian cells. The key challenge for achieving effective gene silencing is primarily dependent on the effectiveness and specificity of the siRNA effector molecule. This is not a trivial matter, as only a limited number of siRNAs are capable of inducing highly effective gene silencing (Hsieh *et al.* 2004; Reynolds *et al.* 2004; Mittal 2004; Naito *et al.* 2009; Petri and Meister 2013). Presently, there is still a lack of clear understanding regarding the properties and factors that determine the gene-silencing efficiency of a given siRNA, albeit that a number of hypotheses have been proposed. These inlcude the thermodynamic properties of the siRNA that determines both its stability and strand bias during duplex unwinding and retention by RISC (Khvorova *et al.* 2003; Lu and Matthews 2008), binding of cellular proteins on the targeted mRNA that may cause positional effects (Miyagishi and Taira 2005), and the local secondary and tertiary structure of the targeted mRNA that may affect the accessibility of the siRNA target sequence (Bohula *et al.* 2003; Gredell *et al.* 2008; Liu *et al.* 2013).

In addition to above, it is also to important to consider that the siRNAs used in this study are validated in mice only. Therefore, there is a strong possibility that the respective siRNAs may display imperfect complementarity to their cognate target mRNAs in BHK-21 cells. In this regard, several reports have been published indicating that mismatches of more than 1 to 2 nt between the guide (antisense) strand of the siRNA and the target mRNA can abolish or diminish siRNA activity (Elbashir *et al.* 2001; Chiu and Rana 2002; Amarzguioui *et al*. 2003; Petri and Meister 2013). It is therefore tempting to speculate that the presence of mismatches between the mouse-validated siRNAs and their target mRNAs in BHK-21 cells could account for the modest level of SARA and UB gene knockdown, as well as the lack of knockdown of Hsp70 gene expression. Furthermore, the imperfect complementarity of a siRNA to its target may lead to translational inhibition, rather than mRNA degradation, by acting as a microRNA (miRNA) (Doench *et al*. 2003; Zeng *et al*. 2003). Due to the lack of SARA-, Hsp70- and UB-

specific antibodies, Western blot analysis could not be performed to investigate this possibility or to determine reductions in the amount of target proteins.

Despite the modest level of knockdown of SARA and UB gene expression, it was next investigated whether suppressing the expression of these cellular genes would influence the release of progeny virions from AHSV-infected cells. For this purpose, BHK-21 cells were transfected with the respective siRNAs and then infected with AHSV-3. The virus remained mostly cell-associated, as typically observed with orbiviruses (Guirakhoo *et al.* 1995), and only minor differences were noted in the percentage virus released from cells treated with mSARA-siRNA and mUB-siRNA (35% to 30%, respectively). However, these differences were not significantly different compared to cells that had been treated with a control non-silencing siRNA (32%). In agreement with its inefficient gene silencing profile, mHsp70-siRNA had no effect on virus release and the results were in agreement with those obtained for the control transfected cells. These results suggest that SARA and UB may not play a role in mediating release of progeny virions from infected cells. However, it is also plausible that these results were due to the inability to reduce the level of target mRNA transcripts to such an extent that it would result in a loss of gene function.

As an alternative to the use of synthetic siRNAs, which is limited by the transient nature of the gene silencing, the use alternative gene knockdown approaches may in future be considered. In this regard, several plasmid and viral vectors have been developed that allow for endogenous expression of small hairpin RNA (shRNAs) bearing a fold-back stem-loop structure that can be converted by Dicer into functional siRNAs. Expression of shRNAs by RNA polymerase III U6 and H1 promoters has been reported to result in efficient and prolonged suppression of targeted genes (Gupta *et al*. 2004; Wadhwa *et al.* 2005; Lambeth and Smith 2013). Indeed, several reports have claimed that endogenously expressed shRNAs are as effective or even more effective than siRNAs to mediate gene silencing (McAnuff *et al.* 2007; Takahashi *et al.* 2009). Should effective RNAi effectors be identified for the SARA, Hsp70 and UB target genes, then this may approach may be useful in especially AHSV-infected cells where long-term gene silencing is required over the course of the experiment. With the advent of next generation sequencing technologies (Metzker 2010), it is no longer implausible that the sequences for the respective target genes could be acquired by sequencing the transcriptome of BHK-21 cells in order to aid in the design of either efficacious siRNAs or shRNAs, which may lead to more effective knockdown in future.

In conclusion, the results obtained in this part of the study indicate that the use of siRNAs, especially in the case of SARA and UB, to silence expression of these genes in infected BHK-21 cells is encouraging. However, further optimization and/or screening of additional candidate RNAi effector molecules from different commercial sources may be required in order to obtain meaningful gene silencing. Furthermore, the host cell proteins interacting with AHSV NS3 were identified by screening cDNA libraries of *Culicoides variipennis* (=*sonorensis*) and *Drosophila melanogaster*. It is therefore possible that such interactions play a relevant role in the non-lytic release of virus particles from insect cells, but are less important during viral egress from mammalian cells. Notably, RNAi has been used in insects such as *Drosophila melanogaster* (Kavi *et al.* 2008; Belles 2010; Muerdter *et al.* 2013) and mosquitoes (Keene *et al*. 2004; Erdelyan *et al.* 2012; Lamacchia *et al.* 2013) to investigate the function of specific genes. Consequently, similar types of studies as those described in this Chapter should be performed in *Culicoides* cells in order to investigate the relevance, if any, of the SARA, Hsp70 and UB proteins in the release of AHSV from insect cells.

CHAPTER 4

CONCLUDING REMARKS

Amongst the proteins encoded by African horsesickness virus (AHSV), the non-structural protein NS3 has been implicated in virus release from infected cells (Stoltz *et al.* 1996; Meiring *et al.* 2009), albeit that the exact mechanism(s) underlying this process is not fully understood. In the case of bluetongue virus, the prototype orbivirus, interaction of the NS3 protein with cellular proteins was shown to facilitate non-lytic release of virions (Beaton *et al.* 2002; Wirblich *et al.* 2006). Interestingly, the results of yeast two-hybrid screens have indicated that the AHSV NS3 protein is capable of interacting with the insect cell proteins SARA, ubiquitin (UB) and Hsp70 (Beyleveld 2007; Barnes 2011). Consequently, the aims of this study were to confirm these interactions using a non-yeast–based assay and to determine whether these cellular proteins may have a functional role in the non-lytic release of AHSV from infected mammalian cells. In this conclusion, the major findings will be summarized briefly and suggestions regarding future research will be made.

Despite the usefulness of yeast two-hybrid screens as a means to identify protein-protein interactions, it is possible that false positive results can be generated and therefore the identified interactions need to be validated in a second non-yeast–dependent system (Serebriiskii *et al.* 2000; Brückner *et al.* 2009). In this study, GST pull-down assays were selected as a means to confirm interaction of the AHSV NS3 protein with the SARA, Hsp70 and UB insect cell proteins. Towards this end, the respective insect cell proteins were expressed successfully as GST fusion proteins and immobilized onto glutathione agarose beads. However, these "bait" proteins were unable to pull down the truncated NS3 protein from *E. coli* BL21(DE3) whole-cell lysates. Based on these results, it may be concluded that NS3 does not interact with the different insect cell proteins and therefore that the interactions determined previously in yeast two-hybrid screens represented false positive interactions. However, such a conclusion may be rash and premature. Note should be taken that the truncated NS3 protein was expressed in low levels in *E. coli* and was only detectable as faint bands by Western blot analyses. It is therefore possible that these low levels of expression may have precluded detection of the truncated NS3 protein by the anti-NS3 antisera under the conditions in which the GST pull-down assays were performed. In future, more sensitive detection methods could be explored such as labelling of the truncated NS3 protein with [^{35}S]-methionine during its synthesis in *E. coli*. This may result in detection of the NS3 protein at levels that might not be detectable with the antibodies used in this study. In

addition, the protein can be detected by autoradiography of the SDS-polyacrylamide gel and therefore would obviate the need to transfer the proteins from the gel to a nitrocellulose membrane for immunological detection, which may result in a reduction in the amount of immobilized protein due to the inefficiency of the transfer process (Towbin *et al.* 1979). Alternatively, the use of other expression systems such as the baculovirus-insect expression system (O'Reilly *et al*. 1992; Hu 2005; Yin *et al*. 2007) may be investigated as a means to obtain higher yields of the truncated NS3 protein for use in the pull-down experiments. It has been reported that the polyhedrin promoter used in this expression system directs high-level heterologous protein synthesis that can represent up to 50% of the total protein of a recombinant baculovirus-infected *Spodoptera frugiperda* host cell (Matsuura *et al*. 1987). Until these approaches or the use of more sensitive *in vitro* and/or *in vivo* validation methods, as discussed previously in Chapter 2, have been explored, the possibility that NS3 interacts with the SARA, Hsp70 and UB insect cell proteins cannot be excluded definitively.

Since the interaction of AHSV NS3 with the different insect cell proteins could neither be confirmed nor disproved, it was next investigated whether knocking down SARA, Hsp70 and UB gene expression may impact on virus egress from infected BHK-21 cells. RNA interference (RNAi) based on the use of exogenously delivered small interfering RNA (siRNA) was used for this purpose. Results obtained during the course of this study indicated that the SARA- and UB-directed siRNAs, in contrast to the Hsp70-directed siRNA, were able to knockdown expression of the respective target genes. However, this had no apparent effect on the release of AHSV from BHK-21 cells since similar levels of virus release were observed in BHK-21 cells that had been transfected with a control non-silencing siRNA. Based on the results, it may thus be tempting to conclude that the SARA and UB proteins are not functionally relevant with regards to a role in AHSV egress. However, there are additional factors that need to be taken into consideration before a definitive conclusion can be drawn. Firstly, the siRNAs used in this study were designed and validated for gene knockdown assays in mice and their efficacy in cultured mammalian cells is not known. Indeed, results obtained in this study indicated that the commercial mHsp70-siRNA was unable to suppress expression of its target gene in BHK-21 cells, whereas the level of knockdown of SARA and UB gene expression was modest (63% and 56%, respectively). Considering that 70% gene knockdown is generally accepted as being meaningful in these types

of studies (Banan and Puri 2004), it is possible that the levels of knockdown achieved in this study were insufficient to effect statistically relevant changes in the release of AHSV from infected BHK-21 cells. Secondly, these experiments were performed in mammalian cells from which AHSV release occurs predominantly through lysis of the infected cells (Guirakhoo *et al.* 1995). It is thus possible that these proteins, especially SARA and UB, play a marginal role during viral egress from the vertebrate host, but may be of importance for the non-lytic release of virions from *Culicoides* insect cells. It therefore follows that RNAi-mediated knockdown studies in AHSV-infected *Culicoides* cells should provide meaningful insights in this regard.

Although the emphasis of this study was on the possible involvement of cellular proteins to facilitate NS3-mediated non-lytic virus release, it is important to note that other viral proteins may also be involved in this process. Previous reports have suggested that the non-structural protein NS1, in conjunction with NS3, may facilitate release of BTV (Owens *et al.* 2004) and AHSV (Meiring *et al.* 2009) from infected cells. Specifically, Owens *et al.* (2004) proposed that the level of NS1 protein relative to the level of NS3 protein in infected cells may dictate whether the progeny virions are released through budding (low level of NS1 relative to that of NS3) or cell lysis (high level of NS1 relative to that of NS3). Therefore, to obtain a more comprehensive picture regarding AHSV egress mechanisms, the involvement of NS1 in virus release should also be investigated in future studies. The use of NS1- and NS3-directed siRNAs as a means to silence expression of the NS1 and NS3 genes individually or in combination may provide new insights regarding the interplay between these two viral proteins and its effect on the mode of virus release.

In summary, further research is required to definitively demonstrate interaction between the AHSV NS3 protein and the SARA, Hsp70 and UB proteins. In addition, the biological relevance of these interactions and their contribution to virus release remains to be determined. Should this be possible, then it may pave the way for a more detailed understanding regarding the mechanism(s) used by AHSV to facilitate virus egress and the role that NS3-host cell protein interactions may play in this process.

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APPENDIX

Nucleotide and deduced amino acid sequence of the SARA gene segment

```
1    G CGG ATC CAG GCC TGG AGT CAA CAC AAT AAA TTG GCT CCA CTG TAC   46
1      R   I   Q   A   W   S   Q   H   N   K   L   A   P   L   Y     15

47     ATG AAT GCC TTA GAT AAT GAA CTT GTA CCG ACA TTA CAT CGT CAA   91
16      M   N   A   L   D   N   E   L   V   P   T   L   H   R   Q    30

92     GCA GGC AAT TTA CAC ATT GAT ACA CCC ATT ATT TTG GAA TTA ATT   136
31      A   G   N   L   H   I   D   T   P   I   I   L   E   L   I    45

137    TTC CAT ATT TTG GAT AAG TGA TAT CCC ACA TTA AAC GGT GAA GCT   181
46      F   H   I   L   D   K   *                                    51

182    TGC                                                           184
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Nucleotide and deduced amino acid sequence of the Hsp70 gene segment

```
1      GCG GGA TCC ATT CGC GGC CGC GTC GAC TCG GAC AAC CAG CCC GGA   45
1       A   G   S   I   R   G   R   V   D   S   D   N   Q   P   G    15

46     GTC TCC ATC CAG GTG TAT GAG GGC GAA CGT GCG ATG ACG AAG GAC   90
16      V   S   I   Q   V   Y   E   G   E   R   A   M   T   K   D    30

91     AAC AAT GCA TTG GGC ACC TTC GAT CTG TCC GGC ATT CCA CCT GCA   135
31      N   N   A   L   G   T   F   D   L   S   G   I   P   P   A    45

136    CCA AGG GGT GTG CCC CAG ATA GAA GTA ACC TTC GAC TTG GAC GCC   180
46      P   R   G   V   P   Q   I   E   V   T   F   D   L   D   A    60

181    AAT GGA ATC CTG AAC GTC AGC GCC AAG GAG ATG AGC ACG GGC AAG   225
61      N   G   I   L   N   V   S   A   K   E   M   S   T   G   K    75

226    GCC AAG AAC ATC ACG ATC AAG AAC GAC AAG GGA CGC CTC TCG CAG   270
76      A   K   N   I   T   I   K   N   D   K   G   R   L   S   Q    90

271    GCC GAG ATT GAT CGC ATG GTG AAC GAG GCT GAG AAG TAC GCC GAC   315
91      A   E   I   D   R   M   V   N   E   A   E   K   Y   A   D    105

316    GAG GAC GAA AAG CAT CGC CAG CGC ATA ACC TCT AGA AAT GCT CTG   360
106     E   D   E   K   H   R   Q   R   I   T   S   R   N   A   L    120

361    GAG AGC TAC GTG TTC AAC GTA AAG CAG TCC GTG GAG CAG GCG CCC   405
121     E   S   Y   V   F   N   V   K   Q   S   V   E   Q   A   P    135

406    GCT GGC AAA CTG GAC GAG GCC GAC AAG AAC TCC GTC CTG GAC AAG   450
136     A   G   K   L   D   E   A   D   K   N   S   V   L   D   K    150

451    TGC AAC GAA ACT ATT CGA TGG CTG GAC AGC AAC ACC ACC GCC GAG   495
151     C   N   E   T   I   R   W   L   D   S   N   T   T   A   E    165

496    AAG GAG GAG TTC GAC CAC AAG ATG GAG GAG CTC ACT CGC CAC TGC   540
166     K   E   E   F   D   H   K   M   E   E   L   T   R   H   C    180

541    TCC CCT ATC ATG ACC AAG ATG CAT CAG CAG GGA GCG GGA GCA GCT   585
181     S   P   I   M   T   K   M   H   Q   Q   G   A   G   A   A    195

586    GGG GGT CCG GGA GCC AAC TGT GGC CAA CAG GCC GGA GGA TTT GGC   630
196     G   G   P   G   A   N   C   G   Q   Q   A   G   G   F   G    210

631    GGC TAC TCT GGA CCC ACA GTC GAG GAG GTC GAC TAA GCA AGC TTG   675
211     G   Y   S   G   P   T   V   E   E   V   D   *                221

676    CG                                                            677
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112

Nucleotide and deduced amino acid sequence of the UB gene segment

```
1   GG ATC CGC GGC CGC GTC GAC CTC ACT GGC AAG ACC ATC ACC TTG GAG   47
1      I   R   G   R   V   D   L   T   G   K   T   I   T   L   E     15

48     GTC GAG CCA TCC GAT ACC ATT GAG AAC GTT AAG GCC AAG ATC CAG    92
16      V   E   P   S   D   T   I   E   N   V   K   A   K   I   Q     30

93     GAC AAG GAG GGA ATC CCC CCA GAT CAG CAG CGT TTG ATT TTC GCC    137
31      D   K   E   G   I   P   P   D   Q   Q   R   L   I   F   A     45

138    GGA AAG CAG CTG GAG GAC GGA CGT ACT CTG TCC GAC TAC AAC ATC    182
46      G   K   Q   L   E   D   G   R   T   L   S   D   Y   N   I     60

183    CAG AAG GAG TCC ACT CTT CAC TTG GTC CTG CGT CTG CGT GGT GGC    227
61      Q   K   E   S   T   L   H   L   V   L   R   L   R   G   G     75

228    ATG CAG ATC TTC GTT AAG ACC CTC ACT GGC AAG ACC ATC ACC TTG    272
76      M   Q   I   F   V   K   T   L   T   G   K   T   I   T   L     90

273    GAG GTC GAG CCA TCC GAT ACC ATT GAG AAC GTT AAG GCC AAG ATC    317
91      E   V   E   P   S   D   T   I   E   N   V   K   A   K   I     105

318    CAG GAC AAG GAG GGA ATC CCC CCA GAT CAG CAG CGT TTG ATT TTC    362
106     Q   D   K   E   G   I   P   P   D   Q   Q   R   L   I   F     120

363    GCC GGA AAG CAG CTC GAG GAC GGA CGC ACT CTG TCC GAC TAC AAC    407
121     A   G   K   Q   L   E   D   G   R   T   L   S   D   Y   N     135

408    ATC CAG AAG GAG TCG ACC CTT CAC TTG GTC CTG CGT CTG CGT GGT    452
136     I   Q   K   E   S   T   L   H   L   V   L   R   L   R   G     150

453    GGC GCC ATC TAG ATT TTT TCA CCC TTA TAA AAA TTG AAG TTA GTT    497
151     G   A   I   *                                                153

498    AGT TGT GCC ACA GGG CAT AAC TCA AGA ATT CAT ACT AAG TTT TAG    542

543    TAA ATT GGT TTA TTC AAA GGC ACA AAA TAA TCA ATC ACA ATA ATT    587

588    ATT GGT ATT CGA AGA ATA AAA TTT TCA ATT TGT GTG TTC AAT GTA    632

633    AGC TTG C                                                     639
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Nucleotide and deduced amino acid sequence of the FLAG-tagged truncated AHSV-3 NS3 gene

```
1    G CAT ATG GAT TAC AAG GAT GAC GAT GAC AAG CTT ATG AGT CTA GCT    46
1          M   D   Y   K   D   D   D   D   K   L   M   S   L   A       14

47     ACG ATC GCC GAA AAT TAT ATG ATG CAT AAT GGA AAT CAG AGA GCA    91
15      T   I   A   E   N   Y   M   M   H   N   G   N   Q   R   A      29

92     ATT GTA CCG TAT GTT CCA CCC CCT TAT GCG TAT GCA AAT GCT CCG    136
30      I   V   P   Y   V   P   P   P   Y   A   Y   A   N   A   P      44

137    ACG CTT GGT GGT CAG GCG GGT GAA ATG GAG TCC ATG TCG CTT GGG    181
45      T   L   G   G   Q   A   G   E   M   E   S   M   S   L   G      59

182    ATA CTT AAT CAA GCC ATG TCA AGT ACA ACT GGT GCA AGT CGG GCT    226
60      I   L   N   Q   A   M   S   S   T   T   G   A   S   R   A      74

227    CTT AAG GAT GAA AAA GCA GCG TTT GGT GCG ATG GCG GAA GCA TTA    271
75      L   K   D   E   K   A   A   F   G   A   M   A   E   A   L      89

272    CGT GAT CCA GAA CCG ATA CGT CAA ATA AAG AAA CAT GTT GGA TTA    316
90      R   D   P   E   P   I   R   Q   I   K   K   H   V   G   L      104

317    AGA ACG CTC AAG CAT TTA AAG ATA GAG TTG GCG TCA ATG TAA CTC    361
105     R   T   L   K   H   L   K   I   E   L   A   S   M   *          117

362    GAG CG                                                         366
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