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Silencing African horsesickness virus VP7 protein expression *in vitro* by RNA interference

by

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Submitted in partial fulfilment of the requirements for the degree of
Master of Science
in the Faculty of Natural and Agricultural Sciences
Department of Microbiology and Plant Pathology
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Pretoria

January 2006

ACKNOWLEDGEMENTS

I would like to express by sincere appreciation to the following people for their contribution towards the completion of this dissertation:

Prof. J. Theron for granting me the opportunity to commence my M.Sc under his supervision.

Prof. H. Huismans for his guidance and interest in my project.

Alan Hall for his assistance in the electron microscopy analysis.

Dr. Michelle van Niekerk for introducing me to cell culture techniques.

The staff and student of the Departments of Microbiology and Plant Pathology and Genetics for their support and guidance during the course of this study.

Flip Wege for cell culture maintenance and titration of the AHSV strains.

Jolene Roos and Marthi Nieuwoudt for their assistance and teamwork on the RNA interference project.

Jacqualine Weyer for supplying me with the enhanced green fluorescent protein gene used in this study.

My family and friends, especially Johann, for their continued support and encouraging attitude towards my work.

The National Research Foundation for financial assistance towards this research.

Dedicated to Izan

SUMMARY

Silencing African horsesickness virus VP7 protein expression in vitro by RNA interference

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African horsesickness virus (AHSV) belongs to the *Orbivirus* genus within the family *Reoviridae*. AHSV is transmitted to vertebrates by *Culicoides* midges and causes an acute disease in horses with a high mortality rate. The virion consists of an outer layer composed of proteins VP2 and VP5, which surround an icosahedral core containing two major proteins, VP3 and VP7, three minor proteins, VP1, VP4 and VP6, and ten segments of double-stranded (ds)RNA. The VP7 protein is not only important in maintaining the structural integrity of the virus particles, but has been reported to play a key role in *Culicoides* cell entry. The phenomenon of RNA interference (RNAi), which can be used to selectively silence homologous genes post-transcriptionally, has revolutionized approaches to study gene function and it is also projected as a potential tool to inhibit virus replication. In mammalian cells, RNAi can be triggered by the direct introduction of 21-23 nucleotide duplexes of small interfering RNA (siRNA) that specifically and potently inhibits endogenous and heterogeneous gene expression. Consequently, the aims of the investigation were to develop RNAi assays whereby the expression of the AHSV-9 VP7

gene could be suppressed in *Spodoptera frugiperda* insect cells and in mammalian BHK-21 cell culture, as well as to determine whether gene-specific siRNAs may prevent AHSV-9 infection in cell culture.

To investigate RNAi-mediated silencing of an enhanced green fluorescent protein (eGFP) in *S. frugiperda* cells, the bacmid expression system was used. Transfection of the insect cells with an eGFP-specific siRNA prior to infection with the recombinant bacmid, inhibited eGFP protein expression by 75%, as quantified by fluorometry. Although the results suggest that RNAi could potentially be used as tool to study the function of an expressed transgene in insect cells, the lack of complete inhibition, coupled with the highly cytolytic nature of the bacmid, may complicate interpretation of the gene interference results.

To investigate whether siRNAs targeting the AHSV-9 VP7 mRNA is able to silence VP7 protein expression, two siRNAs were designed that targeted different regions on the VP7 mRNA. siVP7-336 and siVP7-441, directed at nucleotides 336-356 and 441-461 on the VP7 coding strand, respectively, were chemically synthesized. The effect of these siRNAs on VP7 protein expression was evaluated by cotransfection of BHK-21 cells with the respective siRNAs and the VP7 expression plasmid pCMV-VP7-eGFP. The results indicated that siVP7-336 and siVP7-441 inhibited VP7-eGFP expression by 88% and 75%, respectively. BHK-21 cells were subsequently transfected with the respective siRNAs in separate experiments followed by viral infection. The VP7 mRNA quantities were measured by quantitative reverse transcription PCR and the effect of the siRNAs on viral replication was evaluated by plaque assays. Of the two siRNAs, siVP7-336 was found to be the most effective inhibitor of VP7 transcription and suppressed VP7 mRNA by 93%. The exposure of BHK-21 cells to the VP7 gene-specific siRNA, siVP7-336, also led to a 84% reduction in progeny virions, as measured by a plaque assay. Taken together, the results demonstrate that siRNA-mediated gene silencing is an efficient approach for reducing the level of VP7 transcripts and proteins and for inhibiting virus propagation.

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LIST OF ABBREVIATIONS

AcNPV	*Autographa californica* nuclear polyhedrosis virus
Ago	Argonaute
AHS	African horsesickness
AHSV	African horsesickness virus
Ala	alanine
Arg	arginine
ATP	adenosine-5'-triphosphate
BHK	Baby hamster kidney
BLAST	Basic Local Alignment Search Tool
bp	base pair
BTV	bluetongue virus
˚C	degrees Celsius
ca.	approximately
cDNA	complementary DNA
cm^2	cubic centimetre
CO_2	carbon dioxide
CsCl	cesium chloride
Cys	cysteine
Da	Dalton
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleoside-5'-triphosphate
ds	double-stranded
e.g.	*exempli gratia* (for example)
EDTA	ethylenediaminetetra-acetic acid
eGFP	enhanced green fluorescent protein
EHDV	epizootic haemorrhagic disease virus
et al.	*et alia* (and others)
FBS	foetal bovine serum
FCS	foetal calf serum
Fig.	figure
GAPDH	glyceraldehyde-3-phosphate dehydrogenase

GTP	guanosine-5'-triphosphate
h	hour
i.e.	that is
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase pairs
kDa	kilodalton
kV	kilovolt
L	litre
LB	Luria-Bertani
M	molar
mA	milliampere
MCS	multiple cloning site
MEM	minimum essential medium
mg	milligram
min	minute
miRNA	microRNA
ml	millilitre
mm	millimetre
mM	millimolar
MOI	multiplicity of infection
M_r	molecular weight
mRNA	messenger ribonucleic acid
N	normal
ng	nanogram
nm	nanometer
nt	nucleotides
OD_{600}	optical density at 600 nm
OIE	Office International des Epizootics
ORF	open reading frame
p	probability
p.f.u.	plaque forming units
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pg	picogram
PKR	dsRNA-activated protein kinase R

pmol	picomole
PSB	protein solvent buffer
PTGS	post-transcriptional gene silencing
REST	Relative Expression Software Tool
RGD	arginine-glycine-aspartate
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal RNA
RT	reverse transcription
RT-PCR	reverse transcription-PCR
s	second
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	scanning electron microscopy
shRNA	small hairpin RNA
siGFP-22	eGFP-specific siRNA
siRNA	small interfering RNA
siUN-19	universal negative siRNA
siVP7-336	VP7-specific siRNA targeting nucleotides 336-356
siVP7-441	VP7-specific siRNA targeting nucleotides 441-461
ss	single-stranded
TEMED	N',N',N'-tetramethylethylenediamine
U	units
UHQ	ultra high quality
UTR	untranslated regions
V	volts
v.	version
v/v	volume per volume
VIB	viral inclusion body
w/v	weight per volume
X-Gal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside
β2-MG	β2-microglobulin
µg	microgram

µl	microlitre
µm	micrometre
µM	micromolar
2-5A	2',5'-oligoadenylate synthetase

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CHAPTER ONE

LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

African horsesickness (AHS), of which African horsesickness virus (AHSV) is the causative agent, is a non-contagious, insect-borne disease of *Equidae*. 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 (Mellor and Boorman, 1995; Venter *et al.*, 2000). Although zebras and donkeys rarely exhibit clinical signs of the disease, the effects of the disease, particularly in susceptible populations of horses, can be devastating and mortality rates may exceed 95% (Lord *et al.*, 1997; el Hasnaoui *et al.*, 1998; Fassi-Fihri *et al.*, 1998; Coetzer and Erasmus, 1994). The disease is endemic in sub-Saharan Africa, although outbreaks have occurred in North Africa, the Middle East and in southern European countries (Mellor and Hamblin, 2004). Based on its potential economic and international importance, AHS has been categorized as a list A disease by the Office International des Epizootics (OIE). Such diseases are defined as transmissible diseases that have the potential for very serious and rapid spread, they have particularly serious socio-economic or public health consequences and are of major importance in the international trade of animals and animal products (OIE Manual, 2004).

The continuous expansion of international livestock trade increases the risk of unexpected outbreaks of animal disease. Outbreaks of AHS have a significant economic impact on particularly the international horse trade and horse race industry in disease-free areas, thus great emphasis has been placed on the control of AHS incidence (House, 1998; Sanchez-Vizcaino, 2004). Since AHSV is non-contagious and can only spread via the bites of infected *Culicoides* spp., control may be effected by introducing animal movement restrictions to prevent infected animals from initiating new foci of infection and by slaughtering of viraemic animals to prevent them acting as sources of virus for vector insects (Mellor and Hamblin, 2004). In southern Africa, AHS is controlled by vaccination using polyvalent, live attenuated vaccines that are administered twice in the first and second year of life of susceptible animals, and annually thereafter (Erasmus, 1976; Taylor *et al.*, 1992). However, AHSV-9 and AHSV-5 are not included in the vaccine, since serotypes 9 and 6 are strongly cross-reactive and serotype 5 causes severe reactions, often resulting in death of the vaccinated animals (Erasmus, 1994).

These vaccines are, however, not without their risks and drawbacks. Although the simultaneous inoculation of several vaccine virus types result in the production of protective antibodies against each virus serotype, in some animals, incomplete protection may result from interference between the virus serotypes (Coetzer and Erasmus, 1994) and some vaccine strains may be weakly immunogenic (Laegreid, 1996). Consequently, several courses may be required to achieve full

immunity. Furthermore, even limited replication of AHSV attenuated strains *in vivo* is likely to result in the production of virus-specific antibodies, complicating the distinction between vaccinated and infected animals for import/export purposes (Laviada *et al*., 1995). A further concern is that AHSV has a segmented genome, and consequently, reassortment could occur between live vaccine viruses and field strain viruses, in either the vertebrate or invertebrate host, resulting in viruses with different or enhanced virulence characteristics that express novel antigenic properties (Mellor and Hamblin, 2004). Notably, no AHS vaccines are licensed for use in the European Union (House, 1998). To overcome these disadvantages, a considerable amount of research has been undertaken to develop subunit vaccines based on the use of baculovirus-expressed AHSV-4 VP7 (Wade-Evans *et al*., 1997) and outer capsid proteins (Martinez-Torrecuadrada *et al*., 1996) or BTV core- and virus-like particles (Roy *et al*., 1992; Roy *et al*., 1993; Roy *et al*., 1994a). The subunit vaccines have not yet been commercialized, which could be a reflection of cost and/or difficulties associated with their large-scale production. This warrants that alternative strategies to AHS prevention be investigated.

The baculovirus expression system has been used not only as a means to produce recombinant subunit vaccines, but it has also been used extensively as a means to investigate structure-function relationships of the different AHSV genes and encoded gene products in an effort to elucidate their involvement in the viral replication process (Martinez-Torrecuadrada *et al*., 1994; Roy *et al*., 1997; van Staden *et al*., 1998; Kar *et al*., 2004). However, an exciting new phenomenon, called RNA interference (RNAi), has led to a revolution in molecular biology. RNAi is a eukaryotic mechanism of gene silencing that employs double-stranded (ds) RNA to produce small interfering RNAs (siRNAs) of *ca*. 21 nt in length (Fire *et al*., 1998; Elbashir *et al*., 2001b). A cytoplasmic RNA-induced silencing complex (RISC) binds the siRNA and uses them as guides to direct the degradation of mRNAs containing sequences complementary to one of the strands of the siRNA (Martinez *et al*., 2002; Meister and Tuschl, 2004).

The efficient and sequence-specific nature of RNAi has made it a powerful genetic approach to study the function of mammalian virus genes (Bitko and Barik, 2001; Gitlin and Andino, 2003; López *et al*., 2005). In contrast to currently used gene expression systems, RNAi provides a much more powerful tool to explore and better understand the basic aspects of viral entry into host cells, particle disassembly, replication, particle assembly and export out of the infected cells. In addition to being a valuable research tool, RNAi is also being used as an antiviral strategy in mammalian cells (reviewed in Tan and Yin, 2004). The use of RNAi as an antiviral therapy may have some important advantages. Specific targeting of the viral transcripts and proteins severely impairs viral

replication and promotes eradication of the virus. Furthermore, for gene silencing, only a sub-stoichiometric amount of siRNA is required to drastically decrease mRNA levels. However, the exquisite specificity, without adverse side effects, is the most attractive feature of RNAi as an antiviral approach (Tan and Yin, 2004). Furthermore, as in the case of AHSV, which is transmitted by *Culicoides* spp., RNAi may be used to identify genes involved in vector competence and incidence of transmission (Brown *et al*., 2003). It may in future also be possible to use RNAi as a potential control strategy whereby genetically modified *Culicoides*, by for example transcribing AHSV-specific dsRNA that triggers the RNAi response prior to establishment of infection, could result in poorly competent vector or a vector that completely eliminates the virus infection.

1.2 TAXONOMIC CLASSIFICATION OF AFRICAN HORSESICKNESS VIRUS

AHSV is a member of the genus *Orbivirus* in the family *Reoviridae* (Calisher and Mertens, 1998). The family encompasses viruses with segmented dsRNA genomes (10-12 segments) encapsidated within single non-enveloped virus particles with a diameter of 55-80 nm, which exhibit icosahedral symmetry. In addition to *Orbiviruses*, the other eight genera of this family are *Aquareovirus*, *Coltivirus*, *Cypovirus*, *Fijivirus*, *Phytoreovirus*, *Orthoreovirus*, *Oryzavirus* and *Rotavirus* (Roy, 2001). These viruses have broad host ranges, and have been isolated from a wide variety of terrestrial and non-terrestrial vertebrates and invertebrates, and plants (Francki *et al*., 1991; Gorman, 1992).

The name Orbivirus was proposed by Borden *et al*. (1971) to describe a group of viruses with similar physicochemical properties and morphological appearance, the most characteristic being large ring-shaped capsomeres on the inner shell of the virus particle (Verwoerd, 1969; Verwoerd and Huismans, 1969; Borden *et al*., 1971). The orbiviruses can be distinguished from other members of the *Reoviridae* in that they replicate in both insects and vertebrates (Calisher and Mertens, 1998), show greater sensitivity to lipid solvents and detergents, and virus infectivity is lost in mild acid conditions (Gorman and Taylor, 1985). Within the genus, viruses are divided into 19 distinct serogroups based on cross-reactivities in complement fixation tests, and serotypes within a serogroup are recognized by specific serum-neutralization tests (Gorman, 1979; 1983; 1985; Knudson and Monath, 1990; Brown *et al*., 1991; Calisher and Mertens, 1998). Nine different AHSV serotypes have been distinguished serologically (McIntosh, 1958; Howell, 1962).

1.3 TRANSMISSION, EPIDEMIOLOGY AND PATHOGENESIS OF AFRICAN HORSESICKNESS

AHS, which is endemic in sub-Saharan Africa, is an infectious but non-contagious viral disease of horses. Although zebras have long been considered the natural vertebrate host and reservoir of AHSV (Erasmus *et al*., 1978; Lord *et al*., 1997; Barnard, 1998), antibodies to AHSV have been identified in camels, dogs, cattle, sheep, buffalo, donkeys and mules (Van Rensburg *et al*., 1981; Fassi-Fihri *et al*., 1998; el Hasnaoui *et al*., 1998; Coetzer and Erasmus, 1994). A single incident of AHSV infection in humans by neurotropic strains of the virus (serotypes 1 and 6) has also been reported (Swanepoel *et al*., 1992).

AHSV is transmitted between its equid hosts by biting midges of the genus *Culicoides*, which are the most important vectors of the virus (Wetzel *et al*., 1970; Mellor *et al*., 1975; Mellor, 1993). In Africa, the major vector of AHSV is *C. imicola*, occurring throughout Africa and much of southeast Asia, and southern Europe (Mellor *et al*., 1994; Mellor and Hamblin, 2004). Recent work by Venter *et al.* (2000) has implicated a second African species, *C. bolitinos*, as a potential field vector of AHSV. *C. bolitinos* has a wide distribution in southern Africa and is common in cooler highland areas where *C. imicola* is rare.

AHS is one of the most lethal of equid diseases, and due to its severity, it has been allocated OIE list A status (OIE Manual, 2004). After transfer of the virus by the bite of an infected insect, AHSV is transported to the regional lymph nodes, where initial virus multiplication takes place. This is followed by virus dissemination throughout the body via the blood (primary viraemia). Subsequent infection of target organs and cells, where virus multiplication occurs gives rise to a secondary viraemia (Coetzer and Erasmus, 1994). Four distinct clinical syndromes have been described in horses infected with AHSV, *i.e.* the pulmonary (acute), cardiac (subacute), mixed pulmonary and cardiac (cardio-pulmonary) and fever forms (Erasmus, 1973; Brown and Dardiri, 1990). The fever form of the disease develops after an incubation period of 5-14 days and the affected horses develop mild to moderate fever, scleral infection and mild depression, followed by complete recovery. The cardiac form has an incubation period of 7-14 days and results in mild fever, pericardial effusion and oedema of subcutaneous and intermuscular tissue of the head, neck and chest. Approximately 35% of horses affected with this form of the disease recover, but mortality rates exceeding 50% have been reported. The mixed pulmonary and cardiac form of AHS has symptoms of both forms of the disease and the mortality rate is *ca.* 70% with death occurring within 3-6 days after onset of fever. The pulmonary form develops rapidly (within 4-5 days) and is characterized by high fever,

pulmonary oedema and plural effusion, resulting in a mortality rate often exceeding 95% (Coetzer and Erasmus, 1994).

Until recently, AHS was believed to be confined to sub-Saharan Africa, except for occasional excursion into North Africa or the Arabian Peninsula (Mirchamsy and Hazrati, 1973; Brown and Dardiri, 1992; Mellor, 1994). However, during 1959 and 1961, other countries of the Persian Gulf were affected such as Afganistan, Pakistan, India and most Middle Eastern countries. This region lost over 300 000 animals during this period (Howell, 1960; Mellor and Hamblin, 2004). In 1966, AHS was recognized in Morocco, Algeria, Tunisia and Spain (Mellor and Hamblin, 2004). From 1987-1990, an outbreak of AHS occurred in Spain, Portugal and Morocco, and hundreds of horses died or had to be destroyed (Lubroth, 1988). Of the nine AHSV serotypes, serotypes 1 to 8 are typically found only in restricted areas of sub-Saharan Africa, while serotype 9 is more widespread and has been responsible for virtually all epidemics outside Africa, the exception being the 1987-1990 Spain-Portuguese outbreaks that were due to AHSV-4 (Coetzer and Erasmus, 1994).

1.4 THE STRUCTURE OF AFRICAN HORSESICKNESS VIRUS

Bluetongue virus (BTV) is the prototype virus of the *Orbivirus* genus and has been studied in great detail. Consequently, in the following sections, information on AHSV will be supplemented with that obtained from studies undertaken on BTV.

1.4.1 The viral genome

The genome of AHSV consists of ten dsRNA segments, which vary in size and are numbered 1 to 10 according to their migration in polyacrylamide gels (Oellerman, 1970; Bremer, 1990). The molecular weight of the ten AHSV genome segments range from 2.53 - 0.2 × 10^6 Da, with a total molecular weight of 13 × 10^6 Da (Verwoerd *et al*., 1972; Bremer, 1976; Gorman *et al*., 1977). Each viral segment encodes at least one virus-specific polypeptide (Huismans, 1979; Mertens *et al*., 1984; van Dijk and Huismans, 1988). As is characteristic for the *Reoviridae* family, all ten orbivirus gene segments possess 5'- and 3'-terminal hexanucleotide sequences that are highly conserved in the genus. Orbiviruses additionally have inverted repeat sequences, which differ in sequence for each segment, adjacent to the conserved termini (Rao *et al*., 1983; Cowley *et al*., 1992). The conserved terminal features of the RNA segments are thought to play a role in the determining of mRNA secondary structure that could be important in the sorting and assembly of genome segments during viral replication (Anzola *et al*., 1987; Mizukoshi *et al*., 1993).

1.4.2 The AHSV virion

Three morphologically distinct BTV particles, namely virions, cores and subcore particles have been identified. The AHSV particle is directly comparable to BTV particles both in morphology and molecular constitution (Oellerman *et al*., 1970; Bremer, 1976; Roy *et al*., 1994b) (Fig.1.1). AHSV is a non-enveloped virus containing concentric protein shells. The seven structural proteins (VP1-VP7) are organized into a two-layered protein capsid. The inner capsid (subcore) displays icosahedral symmetry, and is composed of one major protein, VP3, that encloses three minor proteins, VP1, VP4 and VP6, and the ten dsRNA genome segments (Huismans and van Dijk, 1990). Located on this subcore is VP7 (Huismans *et al*., 1987c; Prasad *et al*., 1992), which together with the internal components and subcore form the core. The fully infectious AHSV virion contains an outer capsid that is composed of two major proteins, VP2 and VP5 (Verwoerd *et al*., 1972; Bremer, 1976; Huismans *et al*., 1979).

The overall ultrastructure of the *Orbivirus* genus is typified by that of BTV, the prototype orbivirus. The single- and double-shelled BTV particles have been studied extensively by immunoelectron microscopy and cryo-electron microscopy in conjunction with X-ray crystallography (Hewat *et al*., 1992a; Prasad *et al*., 1992; Grimes *et al*., 1998). Cryo-electron micrographs of the cores confirmed that the structures have a diameter of 69 nm and icosahedral symmetry, with a triangulation number of 13 (Prasad *et al*., 1992). The outer surface of the core is composed of 780 molecules of VP7 (T13) arranged as 260 trimers (Basak *et al*., 1992; Prasad *et al*., 1992; Grimes *et al*., 1995). Channels form between the VP7 trimers and may play a role in the passage of metabolites and viral mRNA into and out of the virus particle during infection (Hewat *et al*., 1992a; Prasad *et al*., 1992; Roy, 1996). The VP7 trimers are also the attachment sites for the two surface proteins, VP2 and VP5. The globular VP5 proteins (360 copies) are situated in the channels formed by the hexameric rings of VP7 trimers, while VP2 proteins (180 copies) form spikes that project beyond VP5 (Hewat *et al*., 1992a; Stuart *et al*., 1998).

Removal of VP7 results in the conversion of core particles to subcores (Huismans *et al*., 1987c). The scaffold of the subcore is composed of the major protein VP3 (120 copies) that is arranged into 60 subunits of two distinct types (A and B), the final folding of which differs slightly from each other. Dimerization occurs such that each dimer consists of two types of VP3 monomers (A and B), and five monomers of VP3A are interconnected to a set of five VP3B monomers. Twelve such decamers are connected together via interaction of the dimerization domain between the decamers to form the subcore structure (Grimes *et al*., 1998; Kar *et al*., 2004). Based on X-ray diffraction

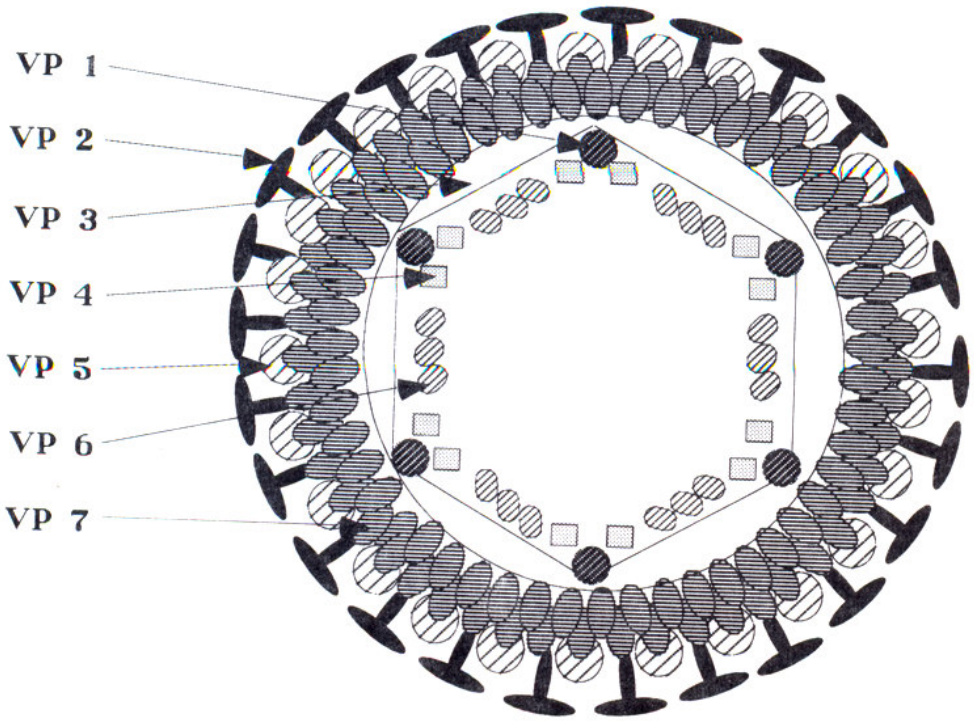

Fig. 1.1 **A schematic diagram of the bluetongue virus particle (Loudon *et al.*, 1992). The outer capsid contains VP2 and VP5 that surrounds the VP7 trimers of the core. VP3 proteins form the scaffold for VP7 trimers and encases the three minor structural proteins VP1, VP4 and VP6, as well as the ten dsRNA genome segments.**

studies, the minor structural proteins are proposed to be attached as a transcriptase complex to the inner surface of the subcore (VP3) at the 5-fold symmetry axis of the decamers (Grimes *et al*., 1998). These complexes may each comprise of 10 or 12 copies of VP1, 20 or 24 copies of VP4 (which forms dimers), and 60 or 72 copies of VP6 (which may form hexamers) per virus particle. In addition, each of the RNA segments may be associated with a transcriptase complex at the 5-fold apices (Stuart *et al*., 1998).

1.4.3 AHSV proteins

The ten AHSV dsRNA segments with their encoded protein products and possible functions are summarized in Table 1.1.

1.4.3.1 The nonstructural viral proteins

The nonstructural viral proteins, NS1, NS2, NS3 and NS3A, are considered to play important roles in the replication and morphogenesis of orbiviruses (Eaton *et al*., 1988; Hyatt *et al*., 1993; van Staden *et al*., 1998) and are often responsible for the formation of characteristic structures in virus-infected cells. In AHSV-infected cells, NS1 and NS2 are synthesized abundantly, and their synthesis coincides with the appearance of two virus-specific structures, namely tubules and granular viral inclusion bodies (VIBs) (Lecatsas, 1968). In contrast, nonstructural proteins NS3 and NS3A are barely detectable in the virus-infected cells (French *et al*., 1989; Wu *et al*., 1992; van Staden *et al*., 1995).

Tubules are entirely composed of the NS1 protein (Huismans and Els, 1979; Maree and Huismans, 1997). Amino acid composition analysis of the NS1 protein indicated that it is particularly rich in Cys residues and contains several hydrophobic and hydrophilic regions spaced throughout the molecule, suggesting it has a highly ordered structure (Maree and Huismans, 1997). Deletion and site-directed mutagenesis of BTV-10 NS1 revealed that cysteine residues at amino acid position 240 and 337, as well as intact amino (N)- and carboxy (C)-terminals of the protein are important for tubule formation (Monastyrskaya *et al*., 1994). The NS1 amino acid sequence, structure and biophysical character of the tubules differ significantly between AHSV and BTV (Huismans and Els, 1979). Whereas BTV tubules have a diameter of 52.3 nm and are up to 1000 nm in length with a helical configuration (Hewat *et al*., 1992b), AHSV tubules have a diameter of 23 nm and vary in length up to 4 µm with a fine "cross-weave" appearance (Maree and Huismans, 1997). Although the function of the NS1 tubules in virus replication is still unclear, it has been proposed initially that they may be involved in the translocation of virus particles from the VIBs to the cell plasma

Table 1.1 AHSV genome segments and their protein products (Adapted from Mertens, 2004)

Segment	No of bp	Encoded protein	No of amino acids	Size (M_r)	Copy number / particle	Location	Function
L1	3965	VP1	1305	150292	10 / 12	Within the subcore	RNA-dependent RNA polymerase
L2	3203	VP2	1051	122043	180	Outer capsid	Serotype-specific antigen, adsorption, neutralization, structural protein involved in determination of virulence
L3	2792	VP3	905	103269	120	Inner capsid	Structural protein, forms scaffold for VP7 trimers, controls overall size and organization of capsid structure
M4	1978	VP4	642	75826	20 / 24	Within the subcore	Capping enzyme, guanylyltransferase
M5	1748	NS1	548	63377	0	Nonstructural protein, forms tubules	Unknown
M6	1566	VP5	504	56900	360	Outer capsid	Structural protein, helps control virus serotype, possible role in membrane penetration during initiation of infection
S7	1167	VP7	349	37916	780	Inner capsid	Group-specific structural protein, possibly involved in cell entry
S8	1166	NS2	365	41193	0	Nonstructural, forms viral inclusion bodies	Binds ssRNA
S9	1169	VP6	369	38464	60 / 72	Within the subcore	Binds ssRNA and dsRNA, helicase, NTPase
S10	756	NS3 NS3A	217 206	23659 22481	0 0	Nonstructural protein in cell membrane	Glycoproteins, membrane proteins, involved in virus release, may be involved in determination of virulence

membrane prior to virus release (Eaton *et al*., 1990), or, alternatively, that they prevent the core particle from assembling before the minor proteins and/or genome segments have been incorporated (Eaton *et al*., 1990; Hewat *et al*., 1992b). However, recent evidence suggests that the BTV NS1 protein is a major determinant of pathogenesis in the vertebrate host since it augments virus-cell association that ultimately leads to lysis of the infected cell (Owens *et al*., 2004).

The NS2 protein has been shown to be a major component of the VIBs observed in orbivirus-infected cells (Thomas *et al*., 1990; Brookes *et al*., 1993). The VIBs have been shown to contain ssRNA, dsRNA, NS1, as well as complete and incomplete virus particles (Eaton *et al*., 1988; Eaton *et al*., 1990; Brookes *et al*., 1993). These observations therefore have led to the recognition of inclusion bodies as the sites in which virus assembly occurs. The expression of BTV and AHSV NS2 alone in the baculovirus expression system results in the formation of VIB-like structures, indicating that NS2 is solely responsible for their formation (Thomas *et al*., 1990; Uitenweerde *et al*., 1995). The NS2 proteins of both BTV and AHSV have been shown to exist as 7S multimers (Uitenweerde *et al*., 1995), which bind ssRNA (Huismans *et al*., 1987b; Uitenweerde *et al*., 1995). It has therefore been suggested that NS2 may play a role in the selection and condensation of the ten viral ssRNA species into precursor subviral particles, prior to dsRNA synthesis. A distinctive feature of orbivirus NS2 is that it is the only virus-specific phosphoprotein (Huismans *et al*., 1987b; Theron *et al*., 1994) and it has been reported that phosphorylation of NS2 down-regulate its ssRNA-binding ability (Theron *et al*., 1994).

In contrast to NS1 and NS2, the two closely related nonstructural proteins NS3 and NS3A are synthesized in low abundance in orbivirus-infected cells (Huismans *et al*., 1979; French *et al*., 1987; van Staden *et al*., 1995). The segment 10 gene, encoding NS3, contains two in-phase translation initiation codons that initiate the synthesis of NS3 and NS3A, respectively (van Staden and Huismans, 1991). Analysis of the deduced amino acid sequence of the segment 10 gene revealed two conserved hydrophobic regions that may serve as transmembrane domains (van Staden and Huismans, 1991; van Niekerk *et al*., 2001). The BTV NS3 protein has been shown to be glycosylated (Wu *et al*., 1992), which may protect NS3 from degradation (Bansal *et al*., 1998). The protein has been found to be associated with intracellular smooth-surface vesicles, as well as with areas of the plasma membrane that have been disrupted by the egress of BTV progeny virions (Hyatt *et al*., 1991; Wu *et al*., 1992). Similarly, the AHSV NS3 protein is an integral membrane protein and is also localized to sites of AHSV release (Stoltz *et al*., 1996). These findings suggest a role for NS3 in the final stages of viral morphogenesis by facilitating the release of progeny virions from infected cells (Hyatt *et al*., 1993; van Staden *et al*., 1995). Moreover, Laegreid *et al*. (1995)

has shown that reassortment and exchange of genome segment 10 between viruses of different serotypes is possible, and that such changes alter the timing of virus release.

1.4.3.2 The core proteins

The three minor proteins, VP1, VP4 and VP6, are candidates for the virus-directed RNA polymerase and associated enzymes, which are responsible for the transcription of the ten viral mRNAs transcribed during infection. The largest AHSV protein, VP1, is considered to be the virus replicase-transcriptase enzyme based on its size (150 kDa), location and molar ratio (estimated at 10-12 molecules per virion) in the core (Stuart *et al*., 1998), and it possesses motifs characteristic of RNA polymerases (Koonin *et al*., 1989; Vreede and Huismans, 1998). Baculovirus-expressed VP1 has been shown to exhibit detectable RNA-elongation activity in the presence of single-stranded poly(U) template and a poly(A) primer (Roy *et al*., 1988; Urakawa *et al*., 1989). During transcription, the mRNA species of BTV are believed to be capped and methylated at their 5'-ends. The VP4 protein of BTV has been reported to bind GTP and has nucleoside triphosphate phosphohydrolase activity (Ramadevi and Roy, 1998), and is thus proposed to be the guanylyl transferase capping enzyme of the virus (Le Blois *et al*., 1992; Ramadevi *et al*., 1998). The third minor protein, VP6, is a highly basic protein with a strong affinity for both ss- and dsRNA (Roy *et al*., 1990; Hayama and Li, 1994; de Waal and Huismans, 2005). Based on its RNA-binding ability, VP6 may be involved in the encapsidation of the RNA (Roy *et al*., 1990; Roy, 1992; Roy *et al*. 1994b). Sequence analysis of VP6 has revealed a motif common to helicases (Roy, 1992; Turnbull *et al*., 1996) and it may therefore also be involved in unwinding the dsRNA genome prior to the initiation of transcription.

The structural proteins VP3 and VP7 are the two major core proteins. Of the two proteins, VP7 is the most abundant and BTV VP7 comprises 36% of the total core protein (Huismans, 1979; Huismans *et al*., 1987c). In both BTV and AHSV, VP7 has been demonstrated to be a serogroup-specific antigen (Huismans and Erasmus, 1981; Chuma *et al*., 1992). In the case of BTV, the protein protrudes through the outer coat of the virus, since it can be detected on the intact particles by anti-VP7 antibodies (Eaton *et al*., 1991). The crystal structure of BTV VP7 has been solved (Basak *et al*., 1992) and the protein was reported to have two domains: a bottom domain (residues 1 to 120 and 250 to 349), which has an all α-helix topology whose base interacts with VP3 in cores; and a top domain (residues 121 to 249), which is an antiparallel β-sandwich. The crystal structure of the top domain of AHSV-4 VP7 has also been solved (Basak *et al*., 1996) and is structurally similar to that of BTV. The top domain of BTV and AHSV VP7 is trimeric and both contain a surface-exposed Arg-Gly-Asp (RGD) tripeptide motif, which, in the case of BTV, has been shown

to be responsible for core attachment to *Culicoides* cells (Tan *et al*., 2001). Notably, in contrast to BTV, AHSV VP7 forms flat hexagonal crystals in the cytoplasm of virus-infected cells (Burroughs *et al*., 1994) and when expressed by a recombinant baculovirus (Chuma *et al.*, 1992). The functional significance of the VP7 crystals, if any, remains to be determined, but it may be possible that these crystals represent a by-product rather than an essential component of the AHSV replication process (Burroughs *et al.*, 1994). The VP3 protein plays a major role in the structural integrity of the virus core and forms the protein scaffold on which the VP7 capsomeres are assembled (Stuart *et al*., 1998). The BTV VP3 protein has been reported to contain group-specific antigenic determinants (Inummaru *et al*., 1987) and is capable of interacting with ssRNA (LeBlois *et al.*, 1992). Co-expression of AHSV VP3 and VP7 genes in insect cells by means of recombinant baculoviruses has been shown to result in their spontaneous assembly into core-like particles (CLPs), which structurally resemble empty authentic AHSV cores (Maree *et al*., 1998).

1.4.3.3 The outer capsid proteins

The VP2 protein, one of the two outer capsid proteins, is the most exposed protein of the virion (Hewat *et al*., 1992a) and therefore is the most variable (Williams *et al*., 1998). Studies with BTV have shown that VP2 is the major serotype-specific antigen (Huismans and Erasmus, 1981) and the viral hemagglutinin (Cowley and Gorman, 1987). Furthermore, VP2 induces neutralizing antibodies that have been shown in the case of BTV to protect sheep against subsequent challenge with the homologous virus serotype (Huismans *et al*., 1987a). Moreover, VP2 is directly involved in the attachment of the virus to cells, since removal of VP2 eliminates binding of the virus to vertebrate cells (Huismans and van Dijk, 1990; Hassan and Roy, 1999). Neutralizing epitopes have been mapped on VP2 (Bremer *et al*., 1990; Venter and Huismans, 1994; Bentley *et al*., 2000; Martinez-Torrecuadrada *et al*., 2001) and antibodies raised in rabbits to AHSV-4 VP2 have been demonstrated to neutralize a virulent strain of AHSV-4 (Martinez-Torrecuadrada *et al*., 1994).

Although present in the outer shell, the other outer capsid protein VP5 is mostly unexposed on the surface of the virus and interacts with the more conserved proteins of the core (Gould and Pritchard, 1988; Wade-Evans *et al*., 1988). The role of VP5 in neutralization has not yet been clearly established. In contrast to BTV (Marshall and Roy, 1990), AHSV VP5 is able to induce neutralizing antibodies, albeit at lower levels than VP2 (Martinez-Torrecuadrada *et al*., 1999). There have been reports indicating that immunization with both VP2 and VP5 elicits higher titres of neutralizing antibody than immunization with VP2 alone, probably by interacting with VP2 and leading to a better exposure of the neutralizing epitopes of VP2 (Marshall and Roy, 1990; Martinez-

Torrecuadrada *et al*., 1996). Consequently, VP5 may play a supportive role to VP2 in enhancing the immune response.

1.5 ORBIVIRUS REPLICATION AND MORPHOGENESIS

Although there are considerable differences in several steps of the replicative process of the *Reoviridae*, the overall strategy is the same. The major events in orbivirus replication are: (i) adsorption and penetration, (ii) uncoating and formation of replicative complexes, (iii) formation of virus tubules and virus inclusion bodies, and (iv) movement of the virus to and release from the cell surface. The following model for orbivirus replication is based on that of BTV, as described by Gould and Hyatt (1994) and Roy (2001).

BTV binds to a receptor(s) of unknown nature in the cell membrane of susceptible host cells. This process is mediated by the outer coat protein VP2, after which the virus enters the cell by endocytosis. Clathrin-coated vesicles, containing the virions, are formed that are drawn to the vicinity of the cell nucleus. The outer capsid proteins (VP2 and VP5) are subsequently removed from the virions in the endosomes, possibly facilitated by the acidic conditions within the endosome, to yield core particles (Huismans *et al*., 1987c), which are released into the cell cytoplasm. The core particles associate with intermediate filaments of the cell cytoskeleton and transcription of the virion RNA occurs. Proteins produced by translation of viral mRNA condense with the viral ssRNA around the parental cores to form VIBs (Eaton *et al*., 1990; Hyatt *et al*., 1992). The mRNA transcripts function not only to encode proteins, but also as templates for production of minus-strands to form the dsRNA genome segments encapsidated in the progeny virions (Mertens and Diprose, 2004).

Since NS2 is the major component of VIBs and is capable of binding ssRNA, it presumably condenses with ssRNA (the predominant form of RNA within VIBs) to form VIBs. Moreover, since assortment of the segments is thought to occur in the VIBs, the NS2 protein may be involved in the condensation of the viral ssRNA segments to the site of assembly and the selection of individual segments on the basis of their identity. However, the exact mechanism and the proteins involved in this process are unknown. During the course of infection, VIBs increase significantly in density and size. Structural proteins are translated on neighboring ribosomes and condense predominantly at the VIB periphery to form subcores and cores. Once these virus structures have been formed, the ssRNA is copied within these structures to form dsRNA (Eaton *et al*., 1990; Hyatt *et al*., 1992; Gould and Hyatt, 1994).

The NS1 protein has been found to be associated with the core-like particles (Eaton *et al*., 1987; Eaton *et al*., 1988). It was subsequently suggested that the addition of NS1 to these particles may prevent transcription of virion RNA by progeny particles lacking VP2 and VP5 or, alternatively, may facilitate the addition of VP2 and or VP5 to the cores (Hyatt *et al.*, 1992). Since the outer capsid protein VP2 is present only on the structurally complete viruses at the edge of the VIBs, it may be inferred that virus particles are assembled at the edge of VIBs and are released into the cytoplasm upon addition of VP2 (Gould *et al*., 1988). However, little is known of the mechanism whereby VP2 and VP5 are added to the developing virus particle and the site of VP5 addition is unknown. The addition of the outer capsid proteins appears to lead to the association of the virus particles with the intermediate filaments of the cell cytoskeleton, which may provide a route whereby progeny viruses are transported to regions of the plasma containing NS3.

BTV may be released from cells in a number of ways (Hyatt *et al*., 1989). The virus may be released from infected cells following the death and subsequent lyses of infected cells. However, viruses are also released as enveloped particles after budding through the host plasma membrane (Gould and Hyatt, 1994) or as non-enveloped virus by extrusion through the membrane (Hyatt *et al*., 1989). When the host cell metabolism is not completely inhibited and the integrity of the host plasma membrane is maintained, elements of the cytoskeleton may push the associated viruses into the neighboring membrane. These particles would be surrounded by the host cell membrane and thus bud from the cell. During the later stages of virus infection, when the integrity of the membrane is not maintained, the virus may push through the membrane (extrusion). The presence of NS3 in host cell membranes where these processes occur indicates a role for the NS3 protein in virus release (Hyatt *et al.*, 1993; Stoltz *et al*., 1996).

1.6 RNA INTERFERENCE (RNAi)

RNA interference (RNAi) is a gene silencing mechanism in which the expression of a gene is specifically inhibited by its cognate dsRNA. Although first described in *Caenorhabditis elegans* (Fire *et al.*, 1998), subsequent studies have shown that RNAi is related to the previously described post-transcriptional gene silencing (PTGS) mechanism of cosuppression in plants (Napoli *et al.*, 1990; Kooter *et al.*, 1999) and quelling in fungi (Cogoni *et al.*, 1996). RNAi appears to be an evolutionary conserved gene silencing system and has been described for protozoa, insects and mammals (Kennerdell and Carthew, 1998; Ngo *et al*., 1998; Wianny and Zernicka-Goetz, 2000). Although the physiological role of RNAi in mammals is yet to be established, it is thought that the natural function of RNAi is to protect the host against transposons (Tabara *et al.*, 1999; Blumenstiel

and Hartl, 2005) and viral infections (Keene *et al.*, 2004; Wilkins *et al.*, 2005), and to maintain normal growth and development (Grishok *et al.*, 2001; Bernstein *et al.*, 2003). Due to its apparent universal applicability, high specificity and simplicity, RNAi is being developed as a tool for analyzing gene functions in diverse groups of organisms and in mammalian and insect cultured cells (Caplen *et al*., 2000; Clemens *et al.*, 2000; Adelman *et al*., 2001; Caplen *et al*., 2001; Elbashir *et al*., 2001a; 2001c; Agrawal *et al*., 2003; Valdes *et al*., 2003). In addition, the replication of a growing number of viruses in cell culture has been inhibited by RNAi, suggesting that it may be developed further into a novel antiviral approach (reviewed in Gitlin and Andino, 2003; Haasnoot *et al*., 2003; Tan and Yin, 2004; Colbère-Garapin *et al*., 2005). Consequently, in this part of the literature review, aspects relating to the molecular mechanism underlying RNAi, as well as the application of RNAi to mammalian cells and its ability to inhibit viral replication will be addressed specifically.

1.7 THE MECHANISM OF RNAi

Based on the results obtained from biochemical and genetic analyses, a two-step mechanistic model for RNAi has been proposed (Agrawal *et al.*, 2003; Meister and Tuschl, 2004). In the first step, referred to as the RNAi initiating step, long dsRNA precursors that vary in length and origin are processed into discreet 21- to 23-nt RNA fragments, which are termed small interfering RNA (siRNA). In the second step, the siRNAs are assembled into RNA-induced silencing complexes (RISC), which degrade the homologous single-stranded mRNA. The model for gene silencing induced by dsRNA is indicated in Fig. 1.2.

1.7.1 Processing of dsRNA

The processing of dsRNA is catalyzed in the cell cytoplasm by a dsRNA-specific RNase III-type endonuclease, termed Dicer (Bernstein *et al*., 2001; Provost *et al.*, 2002), to yield RNA duplexes of *ca.* 21 nt in length, which have 5'-phosphate and 3'-hydroxyl groups and 2-nt 3'overhangs (Tuschl *et al*., 1999; Bernstein *et al*., 2001; Elbashir *et al*., 2001c). Evidence that siRNAs are the true intermediates of the RNAi reaction was initially provided by Zamore *et al.* (2000). Unprocessed dsRNA and processed dsRNA, obtained by making use of an *in vitro* cell-free *Drosophila* system, were fractionated and it was subsequently shown that only fractions containing the native siRNA were able to mediate cognate RNA degradation. Moreover, their ability to degrade RNA was abolished when the siRNAs were denatured at 95˚C for 5 min (Zamore *et al.*, 2000). These results were subsequently confirmed by Elbashir *et al.* (2001c), whom showed that the addition of

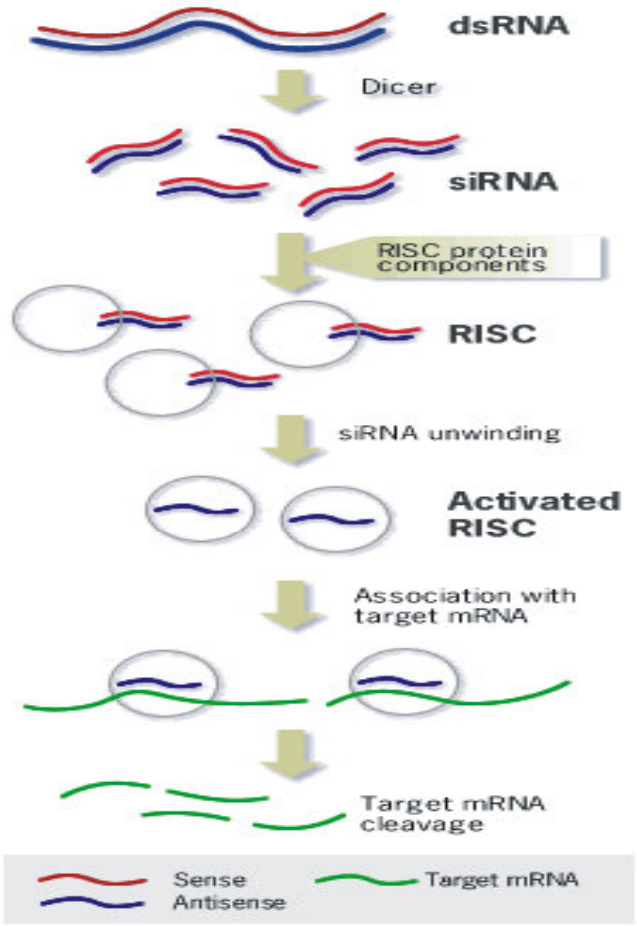

Fig. 1.2 **Two-step model for the mechanism of gene silencing induced by dsRNA. Double-stranded RNA is cleaved by Dicer to produce siRNAs. The siRNAs become associated with the RISC complex, after which the antisense strand guides the complex towards the cognate mRNA, resulting in endonucleolytic cleavage of the mRNA.**

synthetic 21- to 23-nt siRNAs to the *Drosophila* cell-free system was able to guide efficient homologous mRNA degradation. Although Dicer was initially identified in *D. melanogaster* (Bernstein *et al.*, 2001), subsequent studies have shown that *D. melanogaster* has two Dicer genes, *i.e.* DCR-1, which processes miRNA precursors (Lee *et al.*, 2004), and DCR-2, which is required for long dsRNA processing (Pham *et al.*, 2004). The number of Dicer-like proteins varies from one in *C. elegans* and vertebrates to four in *Arabidopsis* (reviewed in Meister and Tuschl, 2004).

Dicers are *ca.* 200-kDa multidomain proteins, which include a DEAH RNA helicase/ATPase domain, dual RNase III domains (RIIIa and RIIIb), a dsRNA-binding domain (dsRBD) and a PAZ domain. The latter is also present in the RDE1/QDE2/Argonaute family of proteins, which has been genetically linked to RNAi (Provost *et al.*, 2002; Tahbaz *et al.*, 2004). In addition to the respective domains, Dicer also interacts with other cellular proteins. Based on results obtained from studying the effects of mutant human Dicer on RNA processing, Zhang *et al.* (2004a) reported that Dicer has only one dsRNA processing center, containing two RNA cleavage sites, and generates products with 2-nt 3' overhangs by cleaving two nearby phosphodiester bonds on opposite RNA strands. It was proposed that Dicer functions through intramolecular dimerization of its two RNase III domains, assisted by the flanking RNA-binding domains, dsRBD and PAZ. Whereas RIIIa cleaves the 3'-hydroxyl-bearing RNA strand, RIIIb cleaves the 5'-phosphate-bearing RNA strand (Zhang *et al.*, 2004a). In contrast to the Dicer of *Drosophila*, recombinant human Dicer has been reported to generate siRNA products from dsRNA efficiently in the presence of Mg^{2+} and the absence of ATP (Zhang *et al.*, 2002).

1.7.2 Assembly of siRNAs into RNA silencing effector complexes

Following cleavage by Dicer, the siRNA-containing ribonucleoprotein particles (RNP) are rearranged into the RNA-induced silencing complex (RISC) (Hammond *et al.*, 2000). The functional RNP contains only single-stranded siRNAs. Every RISC contains a member of the Argonaute protein family, which is thought to bind directly to the single-stranded siRNA residing in RISC (Joshua-Tor, 2004; Martinez and Tuschl, 2004). Several forms of RISC, differing in size and composition, have been reported. The molecular mass ranges from between 130-160 kDa to 500 kDa. The differences in mass appears to be due to weak and/or transient association of proteins involved in the initial processing of dsRNA (Dicer), and other factors of unknown function (Hammond *et al.*, 2001; Nykänen *et al.*, 2001; Martinez *et al.*, 2002; Caudy *et al.*, 2002; Ishizuka *et al.*, 2002).

During the assembly of RISC, ATP is required (Zamore *et al.*, 2000; Bernstein *et al.*, 2001; Nykänen *et al.*, 2001), reflecting the requirement for energy-driven unwinding of the siRNA duplex and/or other conformational or compositional changes of the pre-assembled RNA-duplex-containing RNP. The DEAD-box RNA helicase Armitage of *D. melanogaster* has been shown to be required for RISC assembly (Tomari *et al.*, 2004). Furthermore, the sequence composition of the siRNA duplex appears to have an impact on the ratio of sense and antisense siRNAs entering the RISC complex (Khvorova *et al.*, 2003; Schwarz *et al.*, 2003). Effective siRNAs display reduced thermodynamic stability at the 5'-end of the antisense siRNA relative to the 3'-end within the duplex. The strand bias is presumably caused by a rate-limiting unwinding step that occurs during the transition from the duplex-containing RNP to the larger RISC complex, which allows the 5'-end of the strand positioned at the weakly paired end of the dsRNA to enter RISC first (Khvorova *et al.*, 2003). This property has been exploited in the design of synthetic siRNAs capable of potent gene silencing (Refer to Section 1.8.1).

The Argonaute (Ago) proteins have a molecular mass of 100 kDa and contain two conserved domains: an N-terminal PAZ domain and a C-terminal PIWI domain (Carmell *et al.*, 2002). Recently, it was shown that a subregion of the PIWI domain, namely the PIWI-box, binds directly to the Dicer RNase III domain (Tahbaz *et al.*, 2004). The crystal structure of an Ago protein from the archaeobacterium *Pyrococcus furiosus* has revealed striking similarity of the PIWI domain with members of the RNase H family (Song *et al.*, 2004). Since RNase H cleaves the RNA strand of RNA/DNA duplexes, it was proposed that Ago proteins act by cleaving target RNA in the target RNA/siRNA hybrids (see below). Moreover, the crystal structure of *Archaeoglobus fulgidus* PIWI domain, in association with a siRNA-like duplex, has shown that PIWI specifically recognizes the 5'-end of a siRNA, and unwinds the first base pair of the duplex (Parker *et al.*, 2005). This suggested that binding to the PIWI domain might be the first step in siRNA duplex unwinding in RISC, and PIWI could therefore participate in strand selection. Although the PAZ domain was previously suggested to function as a protein-interaction domain between Ago and Dicer (Hammond *et al.*, 2001), recent biochemical and structural studies have converged on the view that PAZ is a RNA-binding domain that specifically recognizes the terminus of the base-paired helix of siRNA duplexes, including the 2-nt 3' overhang (Lingel *et al.*, 2004; Ma *et al.*, 2004). This highly stable interaction ensures the transfer of siRNA from the RNP complex into RISC by minimizing the possibility of unrelated RNA-processing or RNA-turnover products entering the RNA silencing pathway.

1.7.3 mRNA cleavage

Studies regarding the mechanism of RISC-mediated cleavage have revealed that the single-stranded RISC guides sequence-specific degradation of complementary mRNAs (Zamore *et al*., 2000; Martinez *et al.*, 2002; Martinez and Tuschl, 2004) and that the reaction requires Mg^{2+} ions (Schwartz *et al*., 2004). RISC cleaves the target mRNA in the middle of the complementary region, ten nt upstream of the nucleotide paired with the 5'-end of the guide siRNA (Elbashir *et al*. 2001b; Martinez *et al.*, 2002). The RISC complex catalyzes the hydrolysis of the target-RNA phosphodiester linkage, yielding 5'-phosphate and 3'-hydroxyl termini (Martinez and Tuschl, 2004; Schwarz *et al*., 2004).

It has been proposed on the basis of the similarity of the PIWI domain with RNase H (Song *et al*., 2004) that Ago proteins themselves may act as the catalytic subunit, termed Slicer. The structural similarity includes the carboxylate residues that are thought to bind and position a catabolically important divalent metal ion. The proposed catalytic site lies at the edge of a positively charged groove that extends into the PAZ domain, providing a plausible binding site for the siRNA-substrate duplex. Because RISC and RNase H are both metal-dependent enzymes that cleave one specific strand of a nucleic acid complex and leave chemically similar termini in the products, the structural similarity suggests furthermore that the PIWI domain may harbor the target mRNA cleavage activity of RISC (Song *et al*., 2004). Experimental evidence to this effect, provided by Liu *et al*. (2004b), indicated that although siRNAs bound to all four versions of the human Argonaute proteins, only Argonaute2 was associated with cleavage of target mRNAs. Several site-directed mutations, including two carboxylate residues proposed to bind a catalytic metal ion, within the PIWI domain, specifically blocked cleavage of target mRNAs without affecting Argonaute2 protein expression or siRNA binding. Furthermore, cells cultured from Argonaute2-deficient mice failed to mount an RNAi response upon siRNA transfection (Liu *et al.*, 2004b). Similarly, Parker *et al*. (2005) and Ma *et al.* (2005) both reported that mutagenesis of the putative divalent metal ion-binding site located in the PIWI domain, ablated Slicer activity. However, different organisms have different numbers of Ago proteins and since nuclease activity has thus far only been associated with Argonaute2, it has been proposed that the various Ago proteins may either operate in different forms of RNA silencing (Liu *et al.*, 2004b) or control the extent to which the different RNA silencing systems operate (Meister and Tuschl, 2004).

Recently, the decay pathway of mRNAs targeted by RISC has been investigated in *Drosophila* cells (Orban and Izaurralde, 2005). It was reported that the 5' mRNA fragments generated by RISC cleavage were rapidly degraded from their 3'-ends by the exosome, a multimeric assembly of 3'-to-

5' exonucleases (Parker and Song, 2004). In contrast, the 3' mRNA fragments were degraded from their 5'-ends by XRN1, a major cytoplasmic 5'-to-3' exonuclease (Parker and Song, 2004), and required the Ski complex, which is a heterodimeric protein complex consisting of Ski2p, Ski3p and Ski8p that regulates exosome activity. Furthermore, it was reported that mRNAs targeted by siRNAs are degraded from the ends generated by RISC cleavage, without undergoing decapping or deadenylation (Orban and Izaurralde, 2005).

1.7.4 Amplification and systemic transmission of RNAi

Experiments in *C. elegans* have indicated that only a few copies of dsRNA were required to silence a large excess of target mRNA molecules (Fire *et al.*, 1998). These results suggested that one molecule of dsRNA can promote multiple cycles of mRNA cleavage or that the initial signal is amplified. Subsequent investigations have shown that, in addition to siRNA encoded by the introduced dsRNA, siRNA molecules were identified that lay outside the original targeting area (Sijen *et al.*, 2001). These results indicated that RNAi could spread to adjacent regions and was termed transitive RNAi. Although the underlying mechanism for transitive RNAi is not understood fully, it appears to involve an RNA-dependent RNA polymerase (RdRP) that uses the original siRNA as a primer to form *de novo*-synthesized dsRNA that can subsequently be processed by Dicer into secondary siRNAs and thus amplify the gene-silencing effect in the cells (Sijen *et al.*, 2001, Lipardi *et al.*, 2001). Transitive RNAi has been documented in plants, *C. elegans*, *Neurospora crassa* and *Dictyostelium discoideum*, but not in *D. melanogaster*, mammals or mammalian cell cultures (Stein *et al.*, 2003).

In addition to the amplification of RNAi within one cell, the silencing effect of siRNA can spread throughout the whole organism in worms (Fire *et al.*, 1998). Such systemic RNAi would require a component of the RNAi machinery be passed over the cell membrane to neighboring cells. In *C. elegans*, genetic screening for mutant worms that lacked the systemic response has allowed for the identification of a transmembrane protein, termed SID1 (Winston *et al.*, 2002). It was proposed that SID1 may function as a channel for the import or export of a systemic RNAi signal or might be necessary for endocytosis of the systemic RNAi signal, perhaps by functioning as a receptor. Although no homologue of SID1 has been detected in *D. melanogaster*, which may be consistent with the apparent lack of systemic RNAi in the organism, it has been reported that when SID1 is expressed in *D. melanogaster*, the insect cells were able to take up dsRNA (Feinberg and Hunter, 2003).

1.8 DEVELOPING RNAi FOR USE IN MAMMALIAN CELLS

In *Drosophila*, *S. frugiperda* and *C. elegans*, Dicer processes exogenous dsRNAs into siRNA that subsequently enters the RNAi pathway, resulting in silencing of homologous gene expression (Fire *et al*., 1998; Montgomery *et al*., 1998; Tuschl *et al*., 1999; Valdes *et al*., 2003; Flores-Jasso *et al.*, 2004). In contrast, the introduction of long dsRNA (>30 bp) into the cytoplasm of mammalian cells has been reported to induce the interferon pathway, thus resulting in a systemic, non-specific inhibition of protein synthesis (Manche *et al*., 1992; Stark *et al.*, 1998).

Predominant amongst the responses triggered by the presence of dsRNA in the cell cytoplasm is the activation of two complementary yet independent systems, *i.e.* the dsRNA-activated protein kinase R (PKR) system and the 2',5'-oligoadenylate synthetase (2-5A) system (Stark *et al.*, 1998). PKR is a dsRNA-activated protein kinase that exists in low concentrations in cells in its inactive, unphosphorylated form (Hovanessian, 1989). PKR is activated by binding to dsRNA with a minimal length of 30 bp, but full activation requires dsRNA with a length of 80 bp. Upon binding of dsRNA in a sequence-independent manner, it undergoes autophosphorylation and the activated PKR phosphorylates the alpha subunit of the eukaryotic translation initiation factor elF2, resulting in rapid inhibition of translation (Wang and Carmichael, 2004). In addition, activated PKR also phosphorylates I_kB, releasing it from NF-$_k$B, which is translocated to the nucleus where it activates the expression of the beta-interferon gene. Moreover, PKR can activate apoptotic gene expression and induce apoptosis (Gil and Esteban, 2000; Goodbourn *et al.*, 2000; Li *et al.*, 2003). In interferon-activated cells, expression of 2-5A synthetases, which are encoded by multiple genes and reside in different parts of the cell (Marie and Hovanessian, 1992; Perez *et al*., 1997), are up-regulated. The 2-5A synthetases are activated upon binding to dsRNAs of at least 70 bp, which results in the production of a series of short 2',5'-oligoadenylates, which, in turn, activate the non-specific ribonuclease RNase L. Binding of the 2',5'-oligoadenylates to the inactive monomeric RNase L results in the formation of the homodimeric active RNase L (Kerr and Brown, 1978; Minks *et al.*, 1979), which catalyzes the degradation of single-stranded viral and cellular RNAs, including mRNA, 18S and 28S rRNAs, in a sequence-independent manner (Floyd-Smith *et al.*, 1981; Carroll *et al.*, 1996).

Therefore, in order to apply RNAi technology to studies using mammalian systems, without inducing the dsRNA-activated interferon response, the gene-silencing pathway has to be induced without the use of long dsRNA. This problem was overcome when Elbashir *et al.* (2001a) and Caplen *et al.* (2001) reported that the introduction of synthetic siRNAs of 21 to 22 nt in length, directly into the cytoplasm of mammalian cells, efficiently and specifically silenced expression of

the homologous genes. This discovery opened the door to RNAi approaches in mammalian cells, albeit that the gene silencing was transient. Essentially, application of RNAi to gene silencing in mammalian systems involves careful consideration of the following: (i) selection of siRNA sequences in the target gene, (ii) synthesis and delivery of siRNAs, and (iii) monitoring the efficiency of gene silencing. The evaluation of gene silencing by RNAi generally involves studying the depletion of the target protein by immunofluorescence, immunoblot or reporter protein assays, as well as Northern blot analysis or quantitative real-time RT-PCR to detect specific mRNA degradation of the targeted gene (Caplen *et al*., 2001; Elbashir *et al*., 2001a; Elbashir *et al*., 2002; Ji *et al*., 2003; Park *et al*., 2005). In the following sections, aspects relating to the design, synthesis and specificity of siRNAs will be discussed in greater detail.

1.8.1 Designing an effective siRNA

Several siRNAs synthesized against different regions of the same target mRNA have been reported to show different silencing efficiencies (Elbashir *et al*., 2001a; Harborth *et al*., 2001). Although several parameters for optimizing siRNA-induced gene silencing have been analyzed, including the length, secondary structure, modification of the sugar backbone and sequence specificity, no consensus has evolved on choosing the siRNA sequence (Tuschl *et al.*, 1999; Zamore *et al.*, 2000; Caplen *et al*., 2001; Elbashir *et al*., 2001c). Nevertheless, based on analysis of a small number of target genes that were successfully silenced in *D. melanogaster*, a set of empirical guidelines have been proposed for the design of siRNAs (Caplen *et al.*, 2001; Elbashir *et al.*, 2001b; 2001c). The statistical analyses of increasingly larger groups of sequences have led to the refinement of these guidelines, resulting in substantially improved frequencies whereby functional siRNAs may be obtained (Mittal, 2004; Reynolds *et al.*, 2004; Ui-Tei *et al.*, 2004). In addition, high-throughput screening of potential siRNAs and analysis of effective and ineffective siRNAs, to judge the impact of a number of features on siRNA potency and specificity, has led to the development of several siRNA design algorithms (Amarzguioui and Prydz, 2004). However, these guidelines and algorithms are only predictive and do not guarantee a gene silencing effect. A general rule is that the siRNAs should be 21-nt long and that the sequence of one strand should be AA(N_{19})TT, where N is any nucleotide, *i.e.* the siRNAs should have a 2-nt 3' overhang of uridine residues. In addition, they should have 5'-phosphate and 3'-hydroxyl groups for efficiency (Mittal, 2004). However, siRNAs lacking 3' overhangs (Czauderna *et al.*, 2003a) or siRNAs of 27 nt in length (Kim *et al.*, 2005) have also been reported to be effective in mediating potent and specific inhibition of targeted gene expression in mammalian cells.

The efficiency with which the siRNA interacts with the RISC protein complex and its subsequent unwinding has also attracted much attention, since it appears to be governed partly by the siRNA sequence (Khvorova *et al.*, 2003; Schwarz *et al.*, 2003). The single strand of the siRNA that is incorporated into the RISC protein complex has a lower free energy at the 5'-end when compared to the rest of the siRNA molecule. Khvorova *et al.* (2003) reported that a functional siRNA has a less stable 5' antisense region, but that other factors most likely also contribute to functionality. It seems that RISC attempts to unwind the siRNA from both ends, but that the least stable end is favored. Furthermore, expression-profiling studies have shown that the sense strand of a siRNA can direct off-target gene silencing (Jackson *et al.*, 2003). Therefore, if the siRNA is designed properly, it may be possible to increase the likelihood that the antisense strand, corresponding to the target transcript, can be preferentially incorporated into RISC and thus reduce off-target effects induced by the sense strand (Khvorova *et al.*, 2003; Ui-Tei *et al.*, 2004).

Structural features of the target RNA should, however, also be taken into account when designing siRNAs for gene silencing. Since siRNAs are regarded highly as sequence-specific, it therefore seems reasonable to assume that the local target RNA segment has to be accessible to intermolecular nucleotide-nucleotide interactions with the siRNA. Several reports have suggested that the low activity of some siRNAs may be attributed to non-accessibility of the target mRNA for cleavage, which is caused either by higher order RNA structures (Elbashir *et al.*, 2001a; Bohula *et al.*, 2003; Kretschmer-Kazemi Far and Sczakiel, 2003) or by protein coverage (Elbashir *et al.*, 2001a; Holen *et al.*, 2002). However, the most convincing evidence that target site accessibility dictates the potency of siRNA-mediated silencing has been provided by Brown *et al.* (2005), whom, on the basis of kinetic studies, reported that siRNA-loaded RISC cleaved the target RNA with higher efficiency when target site accessibility was increased.

1.8.2 Synthesis and delivery of siRNAs

The efficient delivery of siRNAs into mammalian cells is a vital step in most RNAi-based gene-silencing experiments and the optimal method whereby siRNAs can be generated often depends on the aims of the experiment. Currently, there are several methods for generating siRNAs, each of which has its own advantages and disadvantages.

1.8.2.1 Synthetic siRNAs

Synthetic siRNA can be obtained either enzymatically or through chemical synthesis. A cost-effective and rapid method for siRNA synthesis is phage T7 RNA polymerase-mediated *in vitro*

transcription from short double-stranded oligonucleotide cassettes that contain the promoter sequence immediately upstream of the siRNA strand template sequence to be transcribed (Donze and Picard, 2002; Sohail *et al.*, 2003). The siRNA strands are synthesized in separate reactions and subsequently hybridized before purification. Since the siRNAs synthesized by this method frequently contain a GGG leader sequence, which is derived from the promoter, and a 5'-triphosphate group (Kim *et al.*, 2004), the hybridized siRNA has to be treated with Tl ribonuclease to remove the single stranded 5'-GGG overhang. Incomplete processing results in the retention of transcripts with 5'-triphosphate groups, which triggers non-specific inhibition of gene expression through the interferon pathway (Kim *et al.*, 2004).

An alternative approach relies on obtaining a pool of enzymatically-generated siRNAs. In this approach, long dsRNA, prepared by *in vitro* transcription, is digested with *E. coli* RNase III or recombinant human Dicer to generate a random array of siRNAs that, upon introduction into cells, is capable of eliciting gene silencing (Yang *et al.*, 2002; Kawasaki *et al.*, 2003; Myers *et al.*, 2003). Although this approach eliminates the need to identify an individual effective siRNA and has been proven to be useful for transiently silencing many endogenous genes in several types of cells, there are some potential problems. Unprocessed or partially processed long dsRNA can activate the dsRNA-dependent protein kinase (PKR), resulting in non-specific translational inhibition and thus requires purification prior to transfection of the siRNA into cells.

In contrast to the above methods, chemically synthesized siRNA are of a uniform composition and can be synthesized at higher amounts and with a wider range of chemical modifications (Chiu and Rana, 2003; Braasch *et al.*, 2003). Although chemical synthesis of siRNA is the most expensive option, it is the most direct approach since the siRNAs can be delivered directly to mammalian cells in culture by electroporation or transfection, thereby resulting in the rapid silencing of target genes (Caplen *et al.*, 2001; Elbashir *et al.*, 2001a; Dykxhoorn *et al.*, 2003; Kishida *et al.*, 2004).

1.8.2.2 Plasmid- or viral vector-expressed siRNAs

An inherent problem associated with siRNA-mediated gene silencing is the variability in transfection efficiency and this is of particular concern when working with difficult-to-transfect cell lines. In addition, the siRNAs induce a transient response, and are therefore not suitable for long-term studies. To overcome these problems, plasmids and viral vectors that stably express RNAi effector molecules have been developed. Different strategies exist for plasmid vector-based RNAi, involving the expression of molecules that can be classified as small hairpin RNA (shRNA) or siRNA. The most commonly used approach, however, involves RNA polymerase III-mediated

transcription of small hairpin structures with a perfectly double-stranded stem of 19 to 29 bp, identical in sequence to the target mRNA, and a short loop of 6 to 9 bases, which is removed *in vivo* by Dicer activity to generate effective shRNAs (Brummelkamp *et al.*, 2002; Paddison *et al.*, 2002a; 2002b; Paul *et al.*, 2002). Thus, for expression of a shRNA, an expression cassette encoding, in the following order, the sense strand of the hairpin, the hairpin loop, the antisense strand of the hairpin, and the terminator, is inserted immediately downstream of the promoter. RNA polymerase III promoters, such as the mouse small nuclear (sn) RNA U6 or human H1 promoters are typically used, since they are active in all cell types and normally transcribe large amounts of small, non-coding transcripts that bear well defined ends (Miyagishi and Taira, 2002; Paddison *et al.*, 2002a; Paul *et al.*, 2002; Sui *et al.*, 2002; Yu *et al.*, 2003). Less commonly, the two strands of a siRNA or shRNA can be transcribed from separate U6 promoters on either the same (Lee *et al.*, 2002) or two separate plasmids (Yu *et al.*, 2002; Wadhwa *et al.*, 2004). Although vector-based RNAi approaches are cost-effective and provide continuous expression of shRNAs over an extended period of time, it has been reported that they can induce an interferon response in mammalian cells (Bridge *et al.*, 2003).

An alternative to the construction of plasmid vectors expressing shRNAs is to generate shRNA expression cassettes (Castanotto *et al.*, 2002; Gou *et al.*, 2003; Scherer *et al.*, 2004). The expression cassettes (SECs) are generated in a two-step PCR of which the final product contains a U6 promoter, followed by the sense strand, a loop, the antisense strand and a terminator sequence. The shRNAs generated by this method are highly specific and efficient, and the suppression of gene expression is comparable to chemically synthesized 21-nt siRNA duplexes or an expression plasmid containing the same shRNA (Castanotto *et al.*, 2002; Gou *et al.*, 2003; Scherer *et al.*, 2004). The disadvantage of this approach is, however, that SECs are difficult to transfect into cells.

The use of viral vectors, such as retroviruses, lentiviruses and adenoviruses, has reportedly allowed siRNA delivery of hard-to-transfect cells. Adenoviruses and adeno-associated viruses (AAV) efficiently transduce many different cell types, including terminally differentiated cells, but they do not integrate into the host genome (Shen *et al.*, 2003; Zhao *et al.*, 2003). In contrast, stable integration of shRNA expression cassettes into the genome of host cells has been achieved by delivery via retroviruses (Brummelkamp *et al.*, 2002; Liu *et al.*, 2004a; Schuck *et al.*, 2004) or lentiviruses (Qin *et al.*, 2003; Rubinson *et al.*, 2003). Lentivirus-mediated delivery has the added advantage over the other systems that it can efficiently integrate into the genome of non-dividing cells, which are refractory to conventional retroviral delivery (Mittal, 2004). In addition to the potential for insertional mutagenesis of chromosomal genes, a further disadvantage associated with

the retrovirus system is the possibility that mutations in the shRNA expression cassette might occur during reverse transcription of the retroviral genome by the error-prone viral reverse transcriptase (Hacein-Bey-Abina *et al.*, 2003). Furthermore, both lentivirus and retrovirus delivery systems have been reported to induce the interferon response, resulting in non-specific gene silencing (Bridge *et al.*, 2003; Fish and Kruithof, 2004).

Although plasmid- and viral vector-based constitutive expression of shRNAs frequently result in stable and efficient suppression of target genes, the inability to adjust levels of suppression has imposed limitations in the analysis of genes essential for cell survival, cell cycle regulation and cell development. Furthermore, suppression of a gene over extended periods can result in physiological responses. These problems have been circumvented by inducible regulation of RNAi in mammalian cells. Tetracycline-inducible H1 (van de Wetering *et al.*, 2003) and U6 (Czauderna *et al.*, 2003b) promoters have been generated by replacement of a 19-bp sequence between the TATA box and the transcription start site with a binding site (tetO) for the tetracycline repressor. In transgenic cells expressing the repressor, repressor binding to the tetO site blocks transcription, while addition of the inducer tetracycline or its derivative, doxycycline, results in dissociation of the repressor, allowing transcription to proceed. A major advantage of the above systems is reversibility of gene suppression, since target gene expression reemerges within 3-4 days of withdrawing the inducer (van de Wetering *et al.*, 2003; Gupta *et al.*, 2004).

1.8.3 Specificity of siRNAs

Currently, siRNAs are being used extensively in mammalian cells to inhibit expression of specific genes. A critical assumption of this approach is that RNAi is sequence-specific, and that the siRNA will selectively inhibit the homologous gene only. The siRNAs are designed to be perfectly complementary to their targets, and it has been reported that mismatches of more than 1 to 2 nt between the antisense strand of the siRNA and the target mRNA could abolish siRNA activity (Tuschl *et al.*, 1999; Elbashir *et al.*, 2001b; Elbashir *et al.*, 2001c; Chiu and Rana, 2002). Several reports have been published indicating that, depending on the position of the mismatch, siRNA activity is affected to different extents. Single mutations within the centre of a siRNA duplex appears to be more discriminating than mutations located at the 5'- and 3'-ends (Amarzguioui *et al.*, 2003; Czauderna *et al.*, 2003a), and in some cases there is enough activity left to mediate significant gene silencing (Holen *et al.*, 2002; Miller *et al.*, 2003).

Some studies have reported that chemically synthesized siRNAs, as well as shRNA produced from plasmid and lentiviral vectors induce the interferon pathway (Bridge *et al.*, 2003; Sledz *et al.*,

2003). However, these effects appear to be concentration dependent and can be overcome by using the lowest effective dose of siRNAs or shRNA-encoding vectors (Bridge *et al.*, 2003; Sledz *et al.*, 2003; Kariko *et al.*, 2004). In addition, siRNAs have been shown to have off-target effects or may also cross-react with targets of limited similarity (Oates *et al.*, 2000; Saxena *et al.*, 2003; Jackson and Linsley, 2004). Consequently, the degree of homology required between the siRNA and its target has been investigated in greater detail and microarray technology was used to measure changes at the mRNA level resulting from siRNA-mediated gene silencing (Chi *et al.*, 2003; Jackson *et al.*, 2003; Semizarov *et al*, 2003; Persengiev *et al.*, 2004). In contrast to Chi *et al.* (2003) and Semizarov *et al.* (2003), whom confirmed the specificity of siRNAs at low concentrations, Jackson *et al.* (2003) and Persengiev *et al.* (2004) reported that the siRNAs were not always target-specific, and observed the direct silencing of non-targeted genes involved in diverse cellular functions. In the study by Persengiev *et al.* (2004), the siRNA used lacked significant sequence similarity to any human gene and it was concluded that the non-specific effects observed on gene expression are dependent upon siRNA concentration. However, Jackson *et al.* (2003) attributed the observed non-specific effects to off-target gene regulation, in which the expression of non-target genes are suppressed due to cross-hybridization of transcripts containing regions of partial homology with the siRNA sequence. These findings could, at least in part, be explained by siRNAs being able to enter the microRNA (miRNA) pathway where perfect homology to the target mRNA is not necessary (Doench *et al.*, 2003). Similarly, Scacheri *et al.* (2004) also observed sequence-dependent non-specific silencing effects at the protein level. Since activation of the dsRNA-triggered interferon pathway did not account for these effects, it was proposed that partial complementary sequence matches to off-target genes may have resulted in a miRNA-like inhibition of translation.

1.9 INHIBITION OF VIRAL REPLICATION BY RNAi

Although the principal application for RNAi-based technologies has thus far been to elucidate the functional role of the genes, it has, however, found increasing application in the prevention and treatment of viral diseases. The first successful attempt to confer antiviral immunity to mammalian cells through the use of RNAi was reported by Bitko and Barik (2001). It was demonstrated that nanomolar concentrations of siRNAs with sequence identity to the phosphoprotein (P) and fusion protein (F) mRNAs, respectively, of respiratory syncytial virus (RSV) caused a significant decrease of viral replication in permissive cells. However, the antiviral potential of RNAi was fully realized when Gitlin et al. (2002) showed that cells transfected with siRNAs targeting the capsid protein- or

the 3D RNA polymerase-encoding regions of the poliovirus genome, prior to virus exposure, resulted in a ca. 100-fold inhibition of virus replication. This study further demonstrated that neither interferon, nor PKR or RNase L were responsible for the suppression of poliovirus replication. Consequently, RNAi has been used to inhibit the replication of numerous mammalian viruses from diverse virus families in vitro and in vivo (Ge et al., 2003; Gitlin and Andino, 2003; Haasnoot et al., 2003; Qin et al., 2003; Bhuyan et al., 2004; Chen et al., 2004; Tan and Yin, 2004; Sanchez-Vargas et al., 2004; Colbère-Garapin et al., 2005).

1.9.1 RNAi-based antiviral strategies

Although it is beyond the scope of this literature review to provide an exhaustive list of viruses targeted by RNAi, the most commonly used approaches whereby viral replication has been inhibited will be addressed and highlighted by a few selected reports. In general, either synthetic siRNAs or vector-expressed shRNAs were used, whilst a few reports have used long dsRNAs to mediate silencing of target genes. In these studies, reductions in virus replication ranging between 2- to 200-fold have been reported.

1.9.1.1 Inhibition of virus replication and/or suppression of viral genome transcription

Of the strategies most frequently used to inhibit virus replication in infected mammalian cells, has been to suppress transcription of the viral genome after entering the host cells or by inhibiting genes that encode proteins that play an essential role during genome replication. RNAi has been shown to block viral replication of the hepatitis B virus (HBV), a member of the *Hepadnaviridae*. The HBV genome is a dsDNA molecule and generates four viral RNAs that encode the core protein (HbeAg) and polymerase-reverse transcriptase, HbsAg, and X protein, respectively. Following cotransfection of HBV and siRNAs against HBV-pregenome RNA into human hepatoma cells, levels of replicative intermediates and viral protein were decreased, indicating that siRNA-mediated gene silencing could inhibit HBV replication through suppression of viral RNA (McCaffrey *et al.*, 2003). In the case of the retrovirus HIV-1, sequences that encode the structural proteins Gag and Env (Hu *et al.*, 2002; Park *et al.*, 2002), as well as the Pol enzyme (Hu *et al.*, 2002) have been targeted for inhibition by RNAi since they are essential for genome transcription. Whereas siRNA-mediated silencing of the respective genes, transfected into the cells either shortly before or after challenge with HIV-1 virions, resulted in a 10-fold inhibition of virus replication (Hu *et al.*, 2002), dsRNAs corresponding to the Gag and Env genes were reported to inhibit virus replication 70-fold (Park *et al.*, 2002). By making use of siRNAs targeting the RNA polymerase of severe acute respiratory syndrome-associated coronavirus (SARS-CoV), Wang *et al.* (2004) demonstrated that

not only were the cytopathic effects of the virus on Vero cells blocked, but that it also blocked viral RNA and protein synthesis, and almost completely inhibited virus production. Similarly, siRNAs directed at two proteins of the transcriptase complex (PA and PB1) of influenza A virus, a member of the *Orthomyxoviridae*, resulted in an overall inhibition of viral RNA transcription *in vitro* (Ge *et al*., 2004).

In contrast to RNA viruses, RNAi against DNA viruses can only result in degradation of the viral mRNAs and not the viral genomes. AcNPV (*Autographa californica* nuclear polyhedrosis virus) is a dsDNA virus of the *Baculoviridae* family that naturally infects many different insect species. Long dsRNAs corresponding to sequences encoding Iel, an early transcriptional activator essential for baculovirus replication, and Gp64, a glycoprotein required for baculovirus entry into host cells by endocytosis, have been transfected into *S. frugiperda* insect cells, and could protect them against subsequent baculovirus infection. In excess of 90% of the cells transfected with dsRNA corresponding to the Iel or Gp64 genes were shown not to be infected with a recombinant baculovirus (Valdes *et al.*, 2003).

1.9.1.2 Targeting viral antigens and cellular factors involved in viral multiplication

RNAi can be used to inhibit the expression of viral antigens and cellular membrane molecules that act as viral receptors, thereby preventing viral entry by blocking the attachment of viruses to surface receptors. Receptors are particularly interesting targets for silencing, because the absence of such receptors can block subsequent steps of the viral infectious cycle and may therefore provide some form of virus resistance. The CD4 molecule is the main receptor for HIV-1, but CCR5, a human chemokine receptor protein, is a necessary coreceptor for infection by most strains of HIV-1. To assess the effect of CD4 and CCR5 silencing on viral entry, siRNA-transfected cells were infected with HIV-1. The results indicated that whereas the expression of CD4 decreased 8-fold and consequently resulted in a 4-fold reduction in viral entry (Novina et al., 2002), a 48% reduction in CCR5 expression resulted in a slight decrease in HIV-1 entry of 2- to 3-fold (Martinez et al., 2002). However, by making use of a lentivirus-based shRNA-expressing system against CCR5 in peripheral blood T-lymphocytes, expression of CCR5 on the cell surface was reduced by 10-fold, resulting in a 3- to 7-fold decrease in the number of infected cells (Qin et al., 2003). Notably, in comparison to direct HIV-1 RNA-targeting (Hu et al., 2002), the indirect silencing of viral receptors appears to be less efficient in reducing virus production. An alternative strategy whereby viral entry into the host cell can be prevented involves targeting viral attachment proteins for degradation. The fusion (F) protein of respiratory syncytial virus (Bitko and Barik, 2001) and spike protein of SARS-CoV (Zhang et al., 2004) each play an important role in viral entry and pathogenesis. Silencing the

expression of these viral attachment proteins by chemically synthesized siRNAs and a shRNA-expressing plasmid vector, respectively, resulted in both cases in a significant reduction in virus titre in cell culture.

1.9.1.3 Interfering with the assembly of viral particles

RNAi may also be used to hinder the assembly of viral particles by silencing expression of viral capsid protein-encoding genes that are typically involved in the assembly of virus particles (Levy *et al*., 1994). Consequently, less infectious viral particles could be obtained and therefore viral infection of susceptible cells may be prevented. In one such study, Déctor *et al*. (2002) targeted the VP4 protein of rotavirus, a member of the *Reoviridae*. The VP4 protein forms part of the three concentric protein layers that encloses the dsRNA genome. It was subsequently shown that in siRNA-transfected cells, followed by virus infection, expression of the VP4 protein was inhibited. In addition, the virus yield was significantly reduced (15-25% of that of a control infection) and the virus particles purified from the siRNA-treated cells were poorly infectious. In a similar study by McCown *et al.* (2003), siRNA specific for the capsid or NS5 open reading frame (ORF) of West Nile virus (WNV), an arbovirus in the *Flaviviridae* family, were transfected into 293T cells, followed by infection of the cells with WNV. It was shown that WNV protein expression, as well as genomic RNA synthesis and infectious virus production were all drastically reduced by siRNAs targeting these two distinct viral sequences.

1.9.2 RNA interference of virus infection *in vivo*

Although the antiviral effect of RNAi in mammalian cells has been demonstrated *in vitro*, as exemplified by the reports above, only a few studies have, however, investigated whether virus replication can be inhibited *in vivo*. Hepatitis B virus (HBV) was the first virus to be inhibited by RNAi *in vivo* in mammals. Inhibition of HBV in the liver of mice was obtained by hydrodynamic transfection of HBV DNA and shRNA-expressing plasmids (McCaffrey *et al.*, 2003), which resulted in a 6-fold decrease in secreted HBV surface antigen in the serum. The murine model has also been used to show that siRNAs specific for highly conserved regions of the nucleoprotein (NP) and acidic polymerase (PA) of influenza A virus inhibit viral replication *in vivo* (Tompkins *et al.*, 2004). In this study, virus-specific siRNAs, administered intranasally, reduced virus titres in the lung and protected mice against lethal challenge with highly pathogenic avian influenza A viruses of the H5 and H7 subtypes. In addition, Ge *et al.* (2004) showed that siRNAs given after influenza virus infection reduced virus replication in the lungs of mice, demonstrating the therapeutic effect of siRNAs. Replication of foot-and-mouth disease virus (FMDV), a highly contagious virus of

livestock and member of the *Picornaviridae* family, has also been inhibited successfully *in vivo*. Chen *et al.* (2004) engineered plasmids encoding capsid protein VP1-specific siRNAs and injected them subcutaneously into the neck of suckling mice. Upon lethal challenge with FMDV, 73-78% of the mice, depending on which VP1-specific siRNA was used, were shown to be protected from infection.

1.10 POTENTIAL CONSTRAINTS TO THE USE OF RNAi AS AN ANTIVIRAL APPROACH

In addition to concerns regarding the delivery and efficacy of different siRNAs and the largely transient nature of RNAi-mediated inhibition of viral replication (Section 1.9), there are, however, a number of other constraints that need to be addressed in order for RNAi to be developed into and used as an antiviral therapeutic agent. These include protection of the viral genomic RNA from siRNA-mediated degradation by viral proteins, the ability of certain viruses to encode suppressor proteins targeted against RNAi and the emergence of virus variants that are apparently refractory to RNAi.

Viral nucleoproteins and/or replication strategies may play a role in shielding the viral genome from the RNAi machinery, thus protecting the viral genomic RNA from degradation. In the case of human respiratory syncytial virus (RSV), it has been reported that the non-segmented genomic and antigenomic RNAs escape siRNAs, possibly because they are tightly associated with the nucleocapsid protein N, which therefore shields them from degradation by the RISC-siRNA complex (Bitko and Barik, 2001). Similarly, in the case of Rous sarcoma virus, the viral mRNA is susceptible to siRNA targeting, but genomic RNA, which is coated by a nucleoprotein, is not (Bitko and Barik, 2001). Recently, Silvestri *et al.* (2004) reported that rotavirus particles, containing all 11 dsRNA genome segments, are formed, despite VP7 or VP4 protein expression having been ablated by siRNAs. It was subsequently proposed that rotavirus plus-strand mRNAs, located outside of the viroplasms (the intracellular sites of virus replication and morphogenesis) and which undergo translation, are susceptible to siRNA-induced degradation. In contrast, plus-strand mRNAs that are located inside viroplasms and undergo replication are not susceptible to RNAi. It may thus be that association of the viral plus-strand mRNAs with the viroplasm, formed by the rotavirus NSP5 nonstructural protein, renders them inaccessible to the RNAi machinery.

It has been reported that mammalian viruses, as with plant viruses, encode suppressor proteins targeted against RNAi (Li *et al*., 2002; 2004b; Lu and Cullen, 2004; Sullivan and Ganem, 2005).

Flock house virus (FHV) encodes a protein, B2, which inhibits RNAi both in *Drosophila* cells and in plant cells, suggesting that it targets a conserved component of the RNAi silencing mechanism (Li *et al.*, 2002). Furthermore, vaccinia virus E3L and influenza virus NS1 proteins have also been reported to be potent suppressors of RNAi in *Drosophila* cells (Li *et al.*, 2004). However, the mechanism by which these proteins inhibit RNAi is not yet known. Moreover, based on the ability of the dsRNA-binding protein σ3 of reovirus to suppress RNAi in plants, presumably by sequestering dsRNAs and thereby inactivating PKR, it has been proposed that virus-encoded dsRNA-binding proteins may also function as inhibitors of RNAi (Lichner *et al.*, 2003). To date, however, only two virus-encoded inhibitors, adenovirus VA1 (Lu and Cullen, 2004) and nodamura virus (NoV) B2 (Sullivan and Ganem, 2005), have been reported to function in mammalian cells. It was shown that adenovirus VA1 RNA directly binds to Dicer and inhibits its function; however, the activity of synthetic siRNA was not affected. In the case of NoV, the B2 protein was reported to bind to pre-Dicer substrate RNA and RISC-processed RNAs, suggesting that it inhibits the Dicer cleavage reaction and, potentially, one or more post-Dicer activities (Sullivan and Ganem, 2005).

Since RNAi relies on nucleotide sequence identity between the siRNA and the target RNA (Tuschl *et al.*, 1999; Caplen *et al.*, 2001; Elbashir *et al.*, 2001c), it can potentially be overcome by point mutations, allowing the possibility of viral escape. This is especially of concern for RNA viruses and retroviruses that exhibit significant genetic variation due to an error-prone replication mechanism. RNAi-resistant poliovirus (Gitlin *et al.*, 2002), HIV-1 (Das *et al.*, 2004) and hepatitis C (Wilson and Richardson, 2005) viruses have been reported. It was shown that these viruses can escape RNAi, following several sequential treatments with the same siRNA, either through the accumulation of multiple nucleotide point mutations within the siRNA target sequence (Wilson and Richardson, 2005), or by modifying the target sequence through nucleotide substitutions or deletions (Gitlin *et al.*, 2002; Das *et al.*, 2004). However, it has been proposed that the emergence of escape mutants may be limited by using multiple siRNAs targeting highly conserved regions of the viral genome (Ji *et al.*, 2003; Liu *et al.*, 2005; Wilson and Richardson, 2005) or, alternatively, by targeting a single (Novina *et al.*, 2002) or multiple (Gitlin and Andino, 2003) host cell factors involved in viral replication.

1.11 AIMS OF THIS STUDY

RNAi has been utilized in numerous organisms, amongst other *C. elegans* (Fire *et al.*, 1998) and *Drosophila* (Kennerdell and Carthew, 1998), and more recently, by expressing siRNA in mammalian cells (Elbashir *et al.*, 2001a) to investigate the function of target genes. Because of its

flexibility, RNAi technology has become a powerful tool for reverse genetic studies in organisms where generating loss-of-function phenotypes by the direct manipulation of genes has proven difficult or impossible. In the case of African horsesickness virus (AHSV), the etiological agent of a highly infectious disease of equines, there is no reverse genetics system available. Thus, the characterization of AHSV gene function has been limited to their expression in prokaryotic and eukaryotic expression systems (Uitenweerde *et al*., 1995; van Staden *et al*., 1995; van Niekerk *et al*., 2001; de Waal and Huismans, 2005). However, the segmented nature of the AHSV genome makes it particularly amenable to analysis by RNAi. Using RNAi technology, it may be possible to silence expression of individual genes without affecting the expression of other viral genes (Déctor *et al*., 2002; López *et al*., 2005). The subsequent analysis of the phenotypes generated should thus allow characterization of the function of proteins encoded by the silenced genes. Consequently, a first step towards such investigations would be to develop and establish RNAi technology for effective and specific silencing of AHSV genes. For this purpose, the AHSV-9 VP7 gene was specifically selected in this study, since it is an important structural determinant of the AHSV particle and is expressed during the late stage of virus infection. In addition to being tool whereby virus gene function can be investigated, RNAi technology has also emerged as a potentially useful method for developing specific gene-silencing therapeutics, especially for the treatment of human and animal viral diseases (McCaffrey *et al.*, 2003; Chen *et al.*, 2004; Tan and Yin, 2004). Since silencing of genes involved in virus particle assembly has been shown to inhibit virus replication (Déctor *et al*., 2002), the AHSV VP7 gene may be an appropriate target in such antiviral approaches.

Therefore, the aims of this investigation were the following:

- To develop an RNA interference (RNAi) assay whereby transgene expression can be silenced in *Spodoptera frugiperda* insect cell culture.

- To develop an RNA interference (RNAi) assay whereby expression of the AHSV-9 VP7 gene can be silenced in mammalian BHK-21 cell culture, using siRNAs designed to target two different regions of the VP7 mRNA.

- To determine whether AHSV-9 replication can be inhibited in siRNA-treated BHK-21 cell culture.

CHAPTER TWO

SMALL INTERFERING RNA-DIRECTED SUPPRESSION OF TRANSGENE EXPRESSION IN *Spodoptera frugiperda* INSECT CELLS

2.1 INTRODUCTION

African horsesickness virus (AHSV), a member of the *Orbivirus* genus in the family *Reoviridae*, is an arthropod-borne virus causing disease in equids, and especially high mortality in horses (Coetzer and Erasmus, 1994). Like other orbiviruses, the genome of AHSV consists of ten double-stranded (ds) RNA segments encoding ten proteins, of which seven are classified as structural proteins (Huismans, 1979; Mertens *et al*., 1984; van Dijk and Huismans, 1988; Bremer *et al.*, 1990; Huismans and van Dijk, 1990; Roy, 2001). The formation and morphology of AHSV particles has been well studied (Grimes *et al*., 1995; Basak *et al*., 1996; Grimes *et al*., 1998; Stuart *et al*., 1998). The inner capsid (core) particle is composed of two major (VP3 and VP7) and three minor (VP1, VP4 and VP6) structural proteins. The core is surrounded by the outer capsid, consisting of two proteins, VP2 and VP5, which is removed when the virus passes through the cell membrane. Not only is the VP7 protein a major inner capsid protein that function principally to maintain the structural integrity of the virion (Williams *et al*., 1998), but also it is a serogroup-specific antigen (Huismans and Erasmus, 1981; Chuma *et al.*, 1992).

Baculovirus expression systems are often used for the expression of biologically active proteins. The first manipulation of a baculovirus for the expression of non-baculoviral DNA was described just over 20 years ago (Smith *et al.*, 1983). Since then, the baculovirus expression system has become one of the most powerful and versatile eukaryotic expression vector systems for heterologous protein expression (O'Reilly *et al.*, 1992; Kost and Condreay, 1999), since it provides a cellular environment that is conducive to the proper folding, disulfide bond formation, oligomerization and/or post-translational modification required for biological activity of some proteins (O'Reilly *et al.*, 1992; Possee, 1997; Kost and Condreay, 1999). The synthesis of individual AHSV proteins by baculovirus recombinants in insect cell culture has made significant contributions to the understanding of AHSV morphology, as well as the function of individual proteins (Uitenweerde *et al.*, 1995; van Staden *et al.*, 1995; Maree and Huismans, 1997; De Waal and Huismans, 2005). In addition, using purified recombinant baculovirus-expressed VP7 protein, the crystal structure of VP7 of AHSV-4 has been resolved (Basak *et al.*, 1996). Furthermore, co-infection of insect cells with VP3 and VP7 recombinant baculoviruses have been reported to result in the intracellular formation of multimeric particles, which structurally resembled authentic AHSV core-like particles (Maree *et al.*, 1998). Thus, by making use of the baculovirus/insect cell expression system studies aimed at understanding the assembly of structural proteins into the mature AHSV particle should be greatly facilitated. Notably, overexpression of AHSV VP7 in insect cells has been reported to result in their aggregation into flat hexagonal crystals (Chuma *et al.*, 1992; Burroughs *et al.*, 1994). Such crystals, which have no apparent functional significance in

the replication cycle of AHSV, is a feature unique to AHSV and has not been observed for other orbiviruses (Basak *et al.*, 1996).

RNA interference (RNAi) is an emerging technology that specifically inhibits gene expression. Small interfering RNA (siRNA), mediators of RNAi, are short (21-23 nt), dsRNA duplexes that inhibit gene expression by inducing gene sequence-specific degradation of homologous mRNA (Fire *et al*., 1998; Montgomery *et al*., 1998; Tuschl *et al*. 1999; Elbashir *et al*., 2001b). These findings raised the possibility that RNAi may not only function as a possible antiviral therapy (Gitlin and Andino, 2003; Tan and Yin, 2004), but also as a tool to explore and better understand aspects relating to, amongst other, viral entry into hosts, particle disassembly and assembly, and export of progeny virions out of the infected cell (Bitko and Barik, 2001; Colbère-Garapin *et al*., 2005; López *et al*., 2005). Many studies have shown that siRNA can significantly suppress gene expression when delivered into mammalian cells *in vitro* (Elbashir *et al*., 2001a; Fish and Kruithof, 2004; Heidel *et al*., 2004; Miller *et al*., 2004; Schuck *et al*., 2004). However, RNAi has also been shown to be very potent in several insect cell lines and adult insects (Tuschl *et al*., 1999; Caplen *et al*., 2000; Hammond *et al.*, 2000; Adelman *et al*., 2001; Valdes *et al*., 2003). It has been reported to function in *Drosophila melanogaster* embryo lysates (Tuschl *et al*., 1999), established cell lines (Caplen *et al*., 2000), as well as in mosquitoes such as *Aedes aegypti, Ae. albopictus*, *Anopheles gambiae* and the malaria vector *An. stephensi* where it exists as an ancient antiviral system (Adelman *et al*., 2001; Caplen *et al*., 2002; Brown *et al*., 2003; Keene *et al*., 2004). In invertebrate cell culture systems, particularly those derived from *D. melanogaster*, *Ae. albopictus* and *Spodoptera frugiperda*, RNAi has been used as a reverse genetics tool for the discovery of novel genes (Ikeda *et al*., 2004; Means *et al.*, 2003) and to study protein function (Clemens *et al.*, 2000; Levashina *et al.*, 2001; Rajagopal *et al.*, 2002; Agrawal *et al*., 2004).

Baculoviruses, in combination with RNAi technology, may provide a potentially powerful tool for gene function exploration. For example, using the baculovirus *Autographa californica* multiple nuclear polyhedrosis virus (AcNPV), Valdes *et al*. (2003) reported that transfection of insect cells with dsRNA from *gp64* and *ie1*, two genes essential for baculovirus replication, resulted in the prevention of viral infection both *in vitro* and *in vivo* upon virus exposure. RNAi was also used to identify a novel gene (*Op-iap3*) from the baculovirus *Orgyia pseudotsugata* MNPV, which was subsequently shown to be required for prevention of apoptosis during virus infection (Means *et al*., 2003). Recently, it was demonstrated that RNAi can be used to study the function of a transgene expressed in insect cells by a recombinant baculovirus (Agrawal *et al*., 2004). In this study, an aminopeptidase N (*apn*) gene, isolated from *S. litura* larvae, was targeted for inhibition by dsRNA

and its function as a receptor for the *Bacillus thuringiensis Cry1C* protein was subsequently established. Since the above reports strongly suggest that RNAi may be a useful tool for identifying and studying the functions of genes from not only baculoviruses, but also from transgenes expressed in the baculovirus/insect cell expression system, the principal aim of this part of the investigation was to develop an RNAi assay for the inhibition of transgene expression *in vitro* in *Spodoptera frugiperda* insect cells. For this purpose, recombinant bacmids expressing either the AHSV VP7 protein, enhanced green fluorescent protein (eGFP) or a chimeric VP7-eGFP protein were engineered, characterized and used towards the establishment of such an assay.

2.2 MATERIALS AND METHODS

2.2.1 Bacterial strains and plasmids

The *E. coli* strains were routinely cultured in LB broth (1% [w/v] tryptone; 1% [w/v] NaCl; 0.5% [w/v] yeast extract; pH 7.4) (Sambrook *et al*., 1989) at 37˚C with shaking at 200 rpm, and maintained at 4˚C on LB agar (LB broth containing 1.2% [w/v] bacteriological agar) or at -70˚C as glycerol cultures. For plasmid DNA selection and maintenance in *E. coli*, the following concentrations of antibiotics were used: 100 µg/ml for ampicillin, 10 µg/ml for tetracycline, 50 µg/ml for kanamycin and 7 µg/ml for gentamicin. All antibiotics were obtained from Roche Diagnostics. Recombinant plasmid pBSS7PCR, containing a full-length cDNA copy of AHSV-9 genome segment 7, was previously constructed and characterized by Mrs S. Maree, Department of Genetics, University of Pretoria. A recombinant pGEM®-T Easy construct containing the enhanced green fluorescent protein (eGFP) gene was obtained from Ms J. Weyer, Department of Microbiology and Plant Pathology, University of Pretoria. The pGEM®-T Easy cloning vector and pFastBac1™ bacmid donor plasmid were obtained from Promega and Invitrogen, respectively.

2.2.2 Plasmid DNA extraction

Plasmid DNA was isolated using the alkaline lysis-method (Birnboim and Doly, 1979), as described by Sambrook *et al.* (1989). For small-scale plasmid extractions, a single colony was inoculated into 5 ml LB broth, containing the appropriate antibiotic and incubated overnight at 37˚C with active aeration (200 rpm). The cells from 3 ml of the overnight cultures were harvested by centrifugation at 15 000 rpm for 1 min and the cell pellets were suspended in 100 µl ice-cold Solution I (50 mM glucose; 10 mM EDTA; 25 mM Tris; pH 8.0), followed by incubation at room temperature for 5 min and 1 min on ice. The resultant spheroplasts were lysed by the addition of 200 µl of freshly prepared Solution II (0.2 N NaOH; 1% [w/v] SDS) and incubated on ice for 5 min, after which the bacterial chromosomal DNA, protein-SDS complexes and high-molecular-weight RNA were precipitated by the addition of 150 µl ice-cold Solution III (3 M NaOAc; pH 4.8). After incubation on ice for 10 min and centrifugation at 15 000 rpm for 10 min, the plasmid DNA was precipitated from the supernatants by the addition of 2.5 volumes 96% ethanol and incubated at -70˚C for 30

min. The plasmid DNA was pelleted by centrifugation at 15 000 rpm for 20 min, rinsed with 70% ethanol, vacuum-dried and then suspended in 30 µl of 1 × TE buffer (10 mM Tris-HCl; 1 mM EDTA; pH 8.0). Contaminating RNA was degraded by the addition of 1 µl RNase A (10 mg/ml), followed by incubation for 30 min at 37˚C.

2.2.3 DNA amplification

2.2.3.1 Oligonucleotides

Oligonucleotides used to amplify the full-length AHSV-9 VP7 and eGFP genes, as well as those used in the construction of a VP7-eGFP chimeric gene, were designed based on nucleotide sequences obtained from sequencing the 5'- and 3'-terminal regions of the respective genes. To facilitate subsequent cloning procedures involving the PCR-amplified products, unique restriction endonuclease recognition sites were included at the 5'-terminus of the different oligonucleotides. The oligonucleotides, indicated in Table 2.1, were synthesized by Inqaba Biotechnical Industries.

2.2.3.2 Polymerase chain reaction (PCR)

Each of the PCR reaction mixtures (50 µl) contained 25-50 ng of template DNA, 100 pmol of each of the sense and antisense oligonucleotides, 1 × PCR buffer (75 mM Tris-HCl [pH 8.8]; 16 mM $(NH_4)_2SO_2$; 0.1% [v/v] Tween-20), $MgCl_2$ at 1.5 mM, each deoxynucleotide triphosphate (dNTP) at a concentration of 250 µM and 2 U of SUPERTHERM *Taq* DNA polymerase (Southern Cross Biotechnology). The tubes were placed in a GeneAmp® 2400 thermal cycler (Applied Biosystems). The thermocycling profile consisted of an initial denaturation step at 94˚C for 3 min, followed by 25 cycles of denaturation at 94˚C for 30 s, annealing at 55˚C for 30 s and elongation at 72˚C for 1.5 min. After the last cycle, the reactions were kept at 72˚C for 5 min to complete synthesis of all DNA strands. For control purposes, reaction mixtures identical to those above were prepared, except that template DNA was omitted. Aliquots of the PCR reaction mixtures were subsequently analyzed by electrophoresis on 0.8% (w/v) agarose gels in the presence of an appropriate DNA molecular weight marker.

2.2.4 Agarose gel electrophoresis

DNA was analyzed by agarose gel electrophoresis. For this purpose, horizontal 0.8% (w/v) agarose gels were cast and electrophoresed at 90 V in 1 × TAE buffer (40 mM Tris-HCl; 20 mM NaOAc; 1 mM EDTA; pH 8.5). The agarose gels were supplemented with 0.5 µg/ml ethidium bromide to allow visualization of the DNA on an UV transilluminator. Where appropriate, DNA fragments were sized according to their migration in the agarose gel as compared to that of standard DNA molecular weight markers, namely phage λ DNA digested with *Hind*III and/or *Eco*RI (Roche Diagnostics), and 100-bp DNA ladder markers, which were obtained from Promega and Fermentas.

Table 2.1 Oligonucleotides used in this part of the study

Oligonucleotides	Nucleotide sequence
PCR amplification*:	
VP7-F	5' - CAC**agatct**ATGGACGCGATAGC - 3'; *Bgl*II site incorporated
VP7-R	5' - CAC**ggtacc**GTAAGTGTATTCGGTATTGAC - 3'; *Kpn*I site incorporated
eGFP-F	5' – CAC**ggatcc**ATGGTGAGCAAGG – 3'; *Bam*HI site incorporated
eGFP-R	5' – CAC**gaattc**TTACTTGTACAGCTCGTCC – 3'; *Eco*RI site incorporated
eGFP-RXho	5' - CAC**ctcgag**TTACTTGTACAGCTCGT - 3'; *Xho*I site incorporated
pHed-F	5' - AAATGATAACCATCTCGC - 3'
Nucleotide sequencing:	
pUC/M13-F	5' - TGTAAAACGACGGCCAGT - 3'
pUC/M13-R	5' - CAGGAAACAGCTATGAC - 3'
VP7-IF	5' - GGCAGATATTACGTACCGCAA - 3'
VP7-RHind	5' - CACAAGCTTGTGGTAGGCTGCTA - 3'
eGFP-IR	5' - TCGGGGTAGCGGCTGAAGCAC - 3'

* In primer sequences, the restriction endonuclease sites are indicated in bold lower case letters.

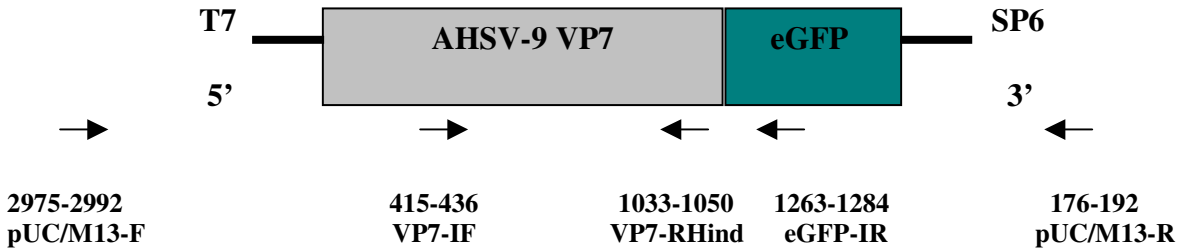

Fig. 2.1 **Schematic representation of the oligonucleotide annealing positions and direction of sequencing of the chimeric AHSV-9 VP7-eGFP gene. The annealing position, indicated above the oligonucleotide, corresponds to its position relative to the first nucleotide of the VP7 initiation codon. The pUC/M13-F and pUC/M13-R oligonucleotides anneal to flanking DNA in the pGEM®-T Easy vector.**

2.2.5 Purification of DNA fragments from agarose gels

DNA fragments were purified from agarose gels using a silica suspension, as described by Boyle and Lew (1995). The appropriate DNA fragment was excised from the agarose gel and mixed with 2.5 volumes of a 6 M NaI solution. The agarose was dissolved by incubating the gel slice at 55˚C for 5-10 min with occasional vortexing, after which 7 µl of a silica suspension was added to the sample. Following incubation on ice for 15 min with intermittent vortexing, the silica-bound DNA was pelleted by centrifugation at 15 000 rpm for 30 s and washed three times with ice-cold NEW wash solution (50 mM NaCl; 10 mM Tris [pH 8]; 2.5 mM EDTA; 50% ethanol). The DNA was eluted from the silica matrix by resuspending the pellet in 7 µl UHQ water and incubating the suspension at 55˚C for 5 min. Following centrifugation at 15 000 rpm for 1 min, the supernatant was collected and 1 µl of the eluate was analyzed by electrophoresis on a 0.8% agarose gel to assess both its purity and concentration.

2.2.6 Cloning of DNA fragments into plasmid vectors

2.2.6.1 Ligation of DNA fragments to vector DNA

PCR amplicons were ligated to pGEM®-T Easy vector DNA (Promega) according to the manufacturer's instructions. Briefly, the gel-purified amplicon (150-200 ng) and pGEM®-T Easy vector DNA (50 ng) were ligated at 15˚C for 16 h in a reaction mixture containing 5 µl of 2 × Rapid Ligation Buffer, 1 µl of T4 DNA Ligase (3 Weiss units/µl) and UHQ water to a final volume of 10 µl. Specific DNA fragments and restricted pFastBac1™ bacmid donor vector DNA were ligated at 15˚C for 16 h in a final volume of 10 µl, which contained 1 µl of T4 DNA ligase (1 U/µl) and 1 µl of a 10 × DNA ligase buffer (Promega). The ratio of vector to insert DNA was typically 1:3.

2.2.6.2 Preparation of competent cells

Competent *E. coli* DH5α cells were prepared and transformed according to the method of Inoue *et al.* (1990), as described by Sambrook and Russel (2001). A starter culture was prepared by inoculating a single colony from a freshly streaked culture of *E. coli* DH5α into 25 ml of SOB broth (10 mM $MgCl_2$; 2.5 mM KCl; 2% [w/v] tryptone; 0.5% [w/v] yeast extract; 0.05% [w/v] NaCl). Following incubation for 6-8 h at 37˚C with shaking (200 rpm), three 1-L Erlenmeyer flasks containing 250 ml of SOB broth were each inoculated with 10 ml, 4 ml and 2 ml of the starter culture, respectively. The cultures were incubated overnight at 18-22˚C with moderate shaking, after which the OD_{600} was measured every 30 min. Once an OD_{600} of 0.55 was reached, the flask was incubated on ice for 10 min to inhibit further growth. The cells were harvested by centrifugation at 4000 rpm for 10 min at 4˚C in a Sorvall centrifuge. The cell pellet was suspended gently in 80 ml of filter-sterilized ice-cold Inoue transformation buffer (10 mM PIPES [pH 6.7]; 55 mM $MnCl_2.4H_2O$; 15 mM $CaCl_2.2H_2O$; 250 mM KCl) and incubated on ice for 10 min. The cells were harvested, as above, and resuspended in 20 ml of Inoue transformation buffer. Following the addition of 1.5 ml of DMSO and incubation on ice for 10 min, the cells were aliquoted into chilled 1.5-ml microfuge tubes and snap-frozen in liquid nitrogen prior to storage at -70˚C.

2.2.6.3 Transformation of competent cells

Prior to transformation, the competent *E. coli* DH5α cells were thawed on ice for 10 min. The cells and ligation reaction mixture (not exceeding 5% of the volume of the competent cells) were mixed and the tubes were maintained on ice for 30 min. After a heat-shock at 42˚C for 90 s, the tubes were chilled on ice for 2 min, and then 900 µl pre-warmed (37˚C) SOC broth (identical to SOB, except for the addition of 20 mM glucose prior to use) was added. A positive control (10 ng of pUC18 plasmid DNA) and negative control (competent cells only) were also included to determine the competency of the *E. coli* DH5α cells and to test for contamination, respectively. The transformation mixtures were incubated at 37˚C for 1 h with agitation before plating aliquots of 100-200 µl onto LB agar supplemented with the appropriate antibiotic. In instances where DNA fragments were cloned into pGEM®-T Easy vector DNA, the cells were plated together with 10 µl of 100 mM IPTG and 50 µl of 2% (w/v) X-Gal to identify recombinant clones based on insertional inactivation of the *lacZ*' marker gene.

2.2.7 Restriction endonuclease digestions

Restriction endonuclease digestions were performed in microfuge tubes in a final volume of 15 µl and contained the appropriate concentration of salt (using the 10 × buffer supplied by the manufacturer) for the specific enzyme and 1 U of enzyme per µg of plasmid DNA. The reaction mixtures were incubated for 1-2 h at 37˚C. Plasmid DNA digested with two enzymes that required different salt concentrations for optimal activity was first digested with the enzyme requiring a lower salt concentration, after which the salt concentration was adjusted and the second enzyme added. All restriction enzymes were supplied by Roche Diagnostics or Promega. The digestion products were analyzed on a 0.8% agarose gel in the presence of appropriate DNA molecular weight markers.

2.2.8 Nucleotide sequencing

The nucleotide sequence of cloned insert DNA was determined using the ABI-PRISM® BigDye™ Terminator v.3.1 Cycle Sequencing Ready Reaction kit (Perkin-Elmer Applied Biosystems) according to the instructions of the manufacturer. In addition to the universal pUC/M13 forward and reverse sequencing oligonucleotides, VP7- and eGFP-specific internal oligonucleotides were also used in the sequencing reactions. The oligonucleotides used in the sequencing reactions are summarized in Table 2.1, whilst a diagram depicting the annealing positions and orientation of the sequencing oligonucleotides are indicated in Fig. 2.1. The sequencing reactions contained 250-500 ng of purified template DNA, 4 µl Terminator Ready Reaction mix, 3.2 pmol sequencing oligonucleotide and UHQ water to a final volume of 10 µl. Cycle sequencing reactions were performed in a GeneAmp® 2400 thermal cycler (Applied Biosystems) with 25 of the following cycles: denaturation at 96˚C for 30 s, annealing at 50˚C for 15 s and extension at 60˚C for 4 min. The extension products were subsequently precipitated by the addition of 2 µl of 3 M NaOAc (pH 4.6) and 50 µl of 95% ethanol to each of the sequencing reactions. The tubes were incubated for 15 min at room temperature in the dark, centrifuged at 15 000 rpm for 30 min and the supernatant carefully aspirated. The pellet was rinsed with 70% ethanol, vacuum-dried and stored at -20˚C until use. Prior to electrophoresis, the

purified extension products were suspended in 3 µl sequencing loading buffer, denatured at 95˚C for 2 min and loaded onto an ABI PRISM® Model 377 DNA sequencer. Nucleotide sequences obtained were analyzed using DNAMAN v.4.13 (Lynnon BioSoft) and BioEdit Sequence Alignment Editor v.5.0.6. (Hall, 1999) software programs.

2.2.9 Plasmid constructions

All molecular cloning techniques employed in the construction of the respective vectors were performed according to the procedures described in the preceding sections. All plasmid constructs were verified by restriction endonuclease digestions and by nucleotide sequencing.

- **pGEM-VP7**

Oligonucleotides VP7-F and VP7-R were used with plasmid pBSS7PCR as template DNA to yield a 1.150-kb amplicon. The VP7 gene-specific amplicon was cloned into pGEM®-T Easy to generate pGEM-VP7.

- **pGEM-eGFP**

Oligonucleotides eGFP-F and eGFP-R were used with plasmid pGEM-eGFPmut as template DNA to generate a 720-bp amplicon, which was cloned into pGEM®-T Easy to generate pGEM-eGFP.

- **pFB-eGFP**

To construct the recombinant pFastBac1™ donor plasmid pFB-eGFP, the eGFP gene was excised from plasmid pGEM-eGFP by digestion with both *Bam*HI and *Eco*RI, and then cloned into pFastBac1™ that had been prepared in an identical manner.

- **pFB-VP7**

To construct recombinant pFastBac1™ donor plasmid pFB-VP7, the AHSV-9 VP7 gene was recovered from plasmid pGEM-VP7 by digestion with both *Bgl*II and *Kpn*I and cloned into pFastBac1™, which had been digested with both *Bam*HI and *Kpn*I.

- **pFB-VP7-eGFP**

A chimeric VP7-eGFP gene was constructed in the mammalian expression vector pCMV-Script® and designated pCMV-VP7-eGFP (Chapter 3). Due to the cloning strategy used it was not possible to recover the VP7-eGFP chimeric gene from pCMV-VP7-eGFP by restriction endonuclease digestion. Thus, the chimeric gene was obtained by PCR amplification using pCMV-VP7-eGFP as template DNA together with oligonucleotides VP7-F and eGFP-RXho (Table 2.1). Following PCR, the amplicon was cloned into pGEM®-T Easy to generate pGEM-VP7-eGFP. To construct the recombinant pFastBac1™ donor plasmid pFB-VP7-eGFP, the VP7-eGFP chimeric gene was recovered by digestion with both *Bgl*II and *Xho*I and then

cloned into pFastBac1™ that had been prepared by digestion with both *Bam*HI and *Xho*I. The cloning strategy employed in the construction of pFB-VP7-eGFP is indicated in Fig. 2.2.

2.2.10 Construction and characterization of recombinant bacmids

2.2.10.1 Preparation and transformation of competent *E. coli* DH10Bac™ cells

An overnight culture of *E. coli* DH10Bac™ cells, containing the bacmid genome and helper plasmid, was prepared by inoculating a single colony in LB broth supplemented with 50 µg/ml kanamycin and 10 µg/ml tetracycline. Competent *E. coli* DH10Bac™ cells (Invitrogen) were prepared, as described previously (Section 2.2.6.2), and then transformed with the recombinant donor plasmids as follows. Competent *E. coli* DH10Bac™ cells (100 µl) were mixed with 10 ng of recombinant donor plasmid DNA in sterile microfuge tubes and incubated on ice for 30 min. The cells were then heat-shocked for 45 s at 42˚C and placed on ice for 2 min before the addition of 900 µl SOC broth. The suspensions were incubated for 4 h at 37˚C to allow the cells to recover and transposition to occur. The transformation mixtures (50-100 µl) were plated onto LB agar supplemented with antibiotics (50 µg/ml kanamycin; 10 µg/ml tetracycline; 7 µg/ml gentamicin), together with 50 µl of 2% (w/v) X-gal and 10 µl of 100 mM IPTG. The agar plates were incubated at 37˚C for at least 24 h. Transformants displaying a white colony-phenotype were restreaked onto fresh LB agar containing antibiotics, as well as X-gal and IPTG to verify the phenotype.

2.2.10.2 Extraction of recombinant bacmid DNA

Colonies confirmed as having a white phenotype were inoculated into 2 ml LB broth containing antibiotics (50 µg/ml kanamycin; 7 µg/ml gentamicin; 10 µg/ml tetracycline) and cultured for 16 h at 37˚C with agitation (200 rpm). The recombinant bacmid DNA was isolated by making use of the following protocol that had been developed for the isolation of large plasmid DNA and was subsequently adapted for the isolation of high-molecular-weight bacmid DNA (Invitrogen). The cells from 1 ml of each culture were collected by centrifugation at 15 000 rpm for 1 min. The supernatant was decanted and the cell pellet suspended in 300 µl of ice-cold Solution I (15 mM Tris-HCl [pH 8.0]; 10 mM EDTA; 100 µg/ml RNase A), followed by the addition of 300 µl of Solution II (0.2 N NaOH; 1% SDS). After incubation at room temperature for 5 min, 300 of µl ice-cold Solution III (3 M KOAc; pH 5.5) was added and the tubes incubated on ice for 10 min. Denatured, insoluble material was removed by centrifugation at 15 000 rpm for 10 min and the bacmid DNA was precipitated from the recovered supernatants by the addition of 800 µl isopropanol. After incubation on ice for 1 h, the precipitated bacmid DNA was collected by centrifugation at 15 000 rpm for 15 min, rinsed with 70% ethanol, air-dried for 10 min at room temperature and then suspended in 40 µl of 1 × TE. The bacmid DNA was analyzed by electrophoresis on a 0.5% (w/v) agarose gel in 1 × TAE buffer at 70 V for 3 h.

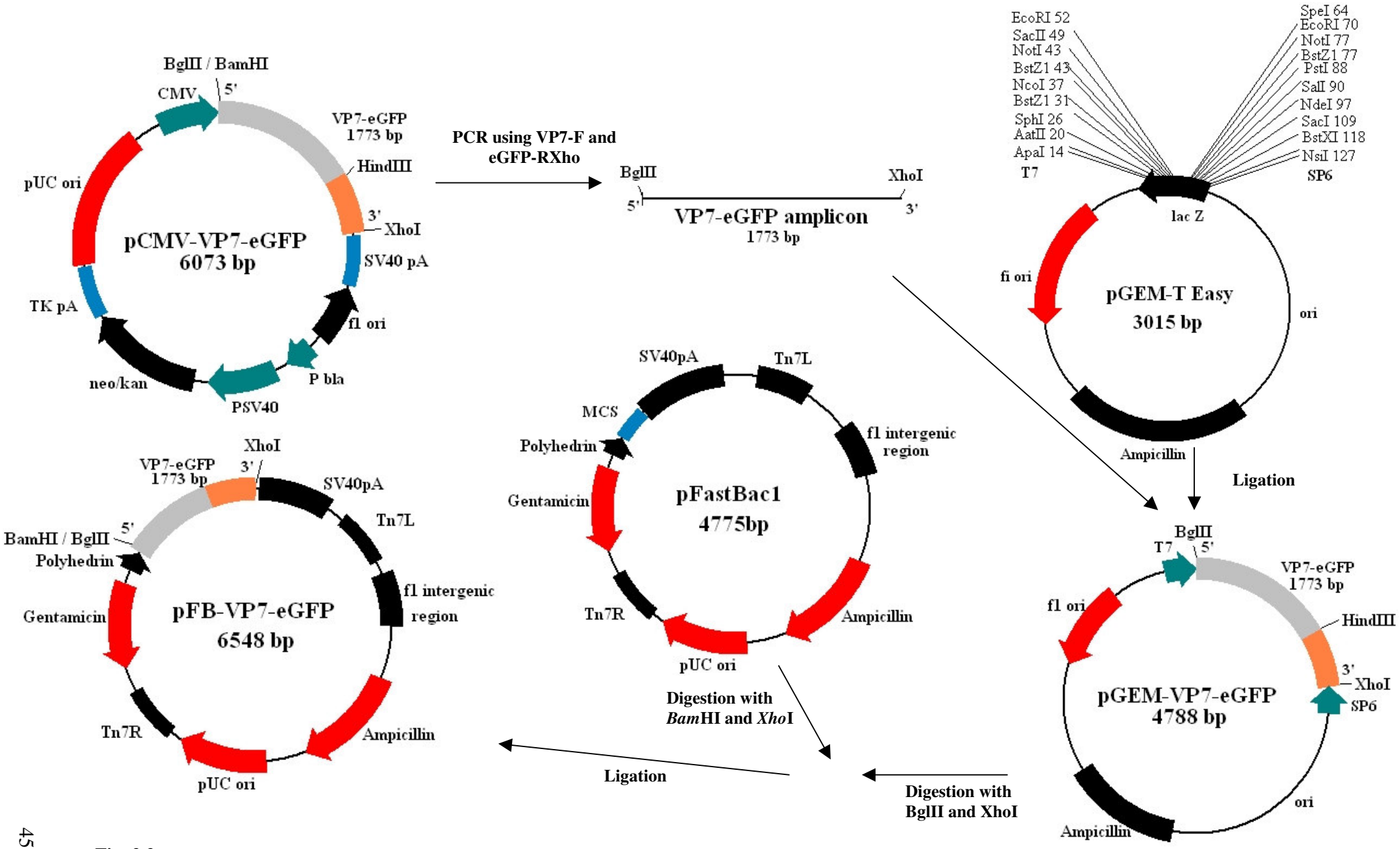

Fig. 2.2 **Construction of the recombinant pFastBac1™ donor plasmid harbouring the AHSV-9 VP7-eGFP chimeric gene.**

2.2.10.3 Analysis of recombinant bacmid

To confirm successful transposition of the eGFP, VP7 and chimeric VP7-eGFP genes into the bacmid DNA, PCR analysis of the extracted bacmid DNA was performed. Each of the reaction mixtures (100 µl) contained 100 ng of bacmid DNA as template, 25 pmol of each oligonucleotides pHed-F and pUC/M13-R (Table 2.1), 1 × PCR buffer (75 mM Tris-HCl [pH 8.8]; 16 mM $(NH_4)_2SO_4$; 0.01% [v/v] Tween-20), 1.5 mM $MgCl_2$, 250 µM of each dNTP and 1.5 U SUPERTHERM *Taq* DNA polymerase (Southern Cross Biotechnology). The PCR was performed using a GeneAmp® 2400 thermal cycler (Applied Biosystems). Following initial denaturation at 93˚C for 3 min, the reaction mixtures were subjected to 30 cycles of denaturation at 94˚C for 45 s, annealing at 55˚C for 45 s and elongation at 72˚C for 5 min. For control purposes, reaction mixtures from which template DNA was omitted, were also included. Aliquots of the reaction mixtures were analyzed by electrophoresis on a 0.8% (w/v) agarose gel in the presence of an appropriate DNA molecular weight marker.

2.2.11 Transfection of *S. frugiperda* cells with recombinant bacmid DNA

2.2.11.1 Cells and culture conditions

Spodoptera frugiperda (Sf-9) cells were propagated as monolayer or as suspension cultures in Spinner flasks at 27˚C, using Grace's insect medium (Highveld Biological) supplemented with 10% (v/v) foetal calf serum (FCS) and the appropriate antibiotics (0.12 mg/ml penicillin; 0.12 mg/ml streptomycin; 0.0325 µg/ml Fungizone) (Highveld Biological). For suspension cultures, the cell density was determined using a haemocytometer. Cultures were seeded at an initial density of 0.2×10^6 cells/ml and subcultured when they reached 2×10^6 cells/ml. Viability of the cells was determined by staining the cells with trypan blue (0.4% [w/v] in 1 × PBS), as described by Summers and Smith (1987).

2.2.11.2 Transfections

The cells were seeded in 35-mm-diameter wells (9×10^5 cells/well) and allowed to attach at room temperature for 1 h. For each transfection, two separate solutions were prepared by diluting 1-2 µg of bacmid DNA and 6 µl of Cellfectin® reagent (Invitrogen) in 100 µl of Grace's medium lacking serum and antibiotics. The two solutions were then combined, mixed gently and incubated at room temperature for 30 min before addition of 800 µl of Grace's medium (lacking serum and antibiotics). In the meantime, the medium was aspirated from the cell monolayers and the cells were washed twice with 2 ml Grace's medium (lacking serum and antibiotics), before being overlaid with 1 ml of the diluted lipid-DNA complexes. The transfected cell monolayers were incubated for 5 h at 27˚C and the transfection mixtures were then removed and replaced with 2 ml Grace's medium supplemented with 10% (v/v) FCS and antibiotics. The dishes were incubated at 27˚C for 96 h, after which the supernatants were removed and stored at 4˚C until use. Mock-transfected cells and cells transfected with wild-type bacmid DNA were included as controls whereby infection of the cells could be monitored.

2.2.11.3 Preparation and titration of virus stocks

To prepare virus stocks, 0.1 ml of the supernatants obtained following transfection of *S. frugiperda* cells was used to infect cell monolayers in 75-cm^2 tissue culture flasks (1×10^7 cells/flask). The supernatants were collected at 72-96 h post-infection, filter-sterilized using a 0.2-µm low protein-binding filter (Cameo 25AS) and then stored at 4˚C. To determine the virus titre, a plaque assay was performed based on the procedures described by Brown and Faulkner (1977). *S. frugiperda* cells were seeded in 35-mm-diameter wells (1×10^6 cells/well) and after adsorption at 27˚C for 1 h at room temperature, the medium was replaced with 900 µl of the virus dilutions (10^{-4} to 10^{-9} prepared in Grace's medium). After incubation at room temperature for 1 h to allow virus particles to infect the cells, the virus dilutions were removed and the cells gently overlaid with 2 ml of sterile 3% (w/v) low melting temperature agarose (LMT) diluted 1:1 in Grace's medium. The tissue culture dishes were incubated at 27˚C for 96 h in a humid environment. The cells were then stained with 0.5 ml Neutral Red (0.1% [w/v] in sterile UHQ water). The excess staining solution was subsequently removed and the dishes were incubated at 27˚C a humid environment until plaques became visible.

2.2.12 SDS-PAGE analysis of bacmid-expressed proteins

2.2.12.1 Preparation of cell lysates

Mock-infected or bacmid-infected *S. frugiperda* cells were harvested from the surface of tissue culture dishes using a syringe, collected by centrifugation at 3 000 rpm for 5 min and washed once in 1 × 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). The cells were suspended in 40-100 µl of 1 × PBS and an equal volume of 2 × protein solvent buffer (PSB: 125 mM Tris-HCl [pH 6.8]; 4% [w/v] SDS; 20% [v/v] glycerol; 10% [v/v] 2-mercaptoethanol; 0.002% [w/v] bromophenol blue) was added to each sample. The samples were heated for 10 min in boiling water prior to SDS-PAGE analysis.

2.2.12.2 SDS-PAGE

Protein samples were analyzed by SDS-PAGE, as described by Laemmli (1970). A 5% (w/v) acrylamide stacking gel and 12% (w/v) acrylamide separating gel was used, of which the acrylamide:bisacrylamide ratio was 30:0.8. The low-porosity separating gel (0.375 M 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 the addition of 0.08% (w/v) ammonium persulphate and 0.008% (v/v) TEMED. The TGS electrophoresis buffer consisted of 0.025 M Tris-HCl (pH 8.3), 0.192 M Glycine and 0.1% (w/v) SDS. Electrophoresis was performed in a Hoefer Sturdier™ SE400 electrophoresis unit for 16 h at 70 V or in a Hoefer Mighty Small™ SE260 electrophoresis unit for 2.5 h at 120 V. After electrophoresis, the gels were stained for 20 min with 0.125% (w/v) Coomassie brilliant blue (prepared in 50% methanol, 10% acetic acid) and the proteins were visualized by counterstaining the gels in a solution containing 25% methanol and 10% glacial acetic acid.

2.2.13 Immunoblot analysis

Immunoblot analysis of the bacmid-expressed proteins was performed, as described by Sambrook *et al.* (1989). Following SDS-PAGE, the gel, two sheets of filter paper and a Hybond™-C$^+$ nitrocellulose membrane (Amersham Pharmacia Biotech AB), cut the same size as the gel, were equilibrated for 30 min in transfer buffer (25 mM Tris; 186 mM Glycine). The proteins were electroblotted onto the membrane for 1.5 h at 28 V and 120 mA, using a Mighty Small™ Transphor blotting apparatus (Hoefer). Following transfer, the gel was recovered and stained with Coomassie brilliant blue to determine the efficiency of the transfer process. The membrane was washed once in 1 × PBS for 5 min and non-specific binding sites were blocked by incubating the membrane at 4˚C overnight in blocking solution (1% [w/v] fat-free milk powder in 1 × PBS). The membrane was transferred to blocking solution containing the primary antibody. These comprised a polyclonal anti-eGFP antibody (Sigma) and an AHSV-9 antiserum (Onderstepoort Veterinary Research Institute), which were diluted 1:1000 and 1:300 in 1 × PBS, respectively. Following incubation at room temperature for 2 h with gentle agitation, the unbound primary antibodies were removed by washing the membrane three times for 5 min each in wash buffer (0.05% [v/v] Tween-20 in 1 × PBS). The secondary antibody, Protein-A conjugated to horseradish peroxidase (Sigma) diluted 1:1000 in 1 × PBS, was added to the membrane and then incubated for 1 h at room temperature. The membrane was washed three times for 5 min each in wash buffer, and once for 5 min in 1 × PBS. To detect immuno-reactive proteins, the membrane was immersed in a freshly prepared enzyme substrate solution (60 mg 4-chloro-1-naphtol [Sigma] in 20 ml ice-cold methanol and 60 µl H_2O_2 in 100 ml 1 × PBS, mixed just before use). Once the bands became visible, the membranes were rinsed with distilled water and air-dried.

2.2.14 Characterization of bacmid-expressed proteins

2.2.14.1 Sedimentation analysis

Monolayers of *S. frugiperda* cells (1 × 10^7 cells/75-cm^2 flask) were infected at a MOI of 5 p.f.u./cell with the recombinant bacmids and incubated at 27˚C for 96 h. The cells were subsequently harvested, collected by centrifugation at 3 000 rpm for 5 min and washed once with 10 ml of 1 × PBS. The cell pellet was suspended in 1 ml of 1 × PBS, homogenized by 15-18 strokes with a Dounce homogenizer and centrifuged at 1000 rpm for 1 min. An aliquot (500 µl) of the cytoplasmic extract was loaded onto a 50-70% linear sucrose gradient (prepared in 1 × PBS). The gradients were centrifuged at 40 000 rpm for 18 h at 4˚C in a Beckman SW 55Ti rotor, using a Sorvall® Ultra Pro80 centrifuge (DuPont). Twelve fractions of 20 drops each (*ca.* 400 µl/fraction) were collected dropwise from the bottom of the gradients. The pellets were suspended in 40 µl of 1 × PBS. The proteins from each fraction were recovered by diluting 200 µl of each fraction with 1.2 ml 1 × PBS, followed by centrifugation at 5 000 rpm for 45 min at 4˚C. The protein pellets were suspended in 15 µl 1 × PBS and analyzed by SDS-PAGE analysis.

2.2.14.2 Scanning electron microscopy (SEM)

For SEM, the sucrose gradient-purified protein samples were first fixed for 1 min at room temperature in 0.75 M NaH_2PO_4 buffer containing 0.1% (v/v) gluteraldehyde and then filtered through a 0.22-µm filter (Millipore). The filter-bound proteins were washed six times, 10 min each wash, in 0.075 M NaH_2PO_4 before being dehydrated by sequential treatment for 10 min each in 50%, 70%, 90% and 100% ethanol. The treatment with 50-90% ethanol was performed twice, whilst treatment with 100% ethanol was repeated six times to ensure complete dehydration of the samples. The samples were critical point-dried, placed onto an aluminium stub, scatter-coated with gold and observed in a JEOL840 scanning electron microscope at 5 kV.

2.2.15 RNA interference assays in *S. frugiperda* cells

2.2.15.1 Small interfering RNAs (siRNAs)

A chemically synthesized siRNA directed against eGFP mRNA, which has been reported to inhibit eGFP expression by 90% (Caplen *et al.*, 2001), was obtained from Qiagen. In addition, a negative control siRNA was designed by scrambling the sequence of the eGFP. The sequences used were as follows: siGFP-22, sense 5'-GCAAGCUGACCCUGAAGUUCAU-3', antisense 5'-GAACUUCAGGGUCAGCUUGCCG-3'; and scrambled siRNA, sense 5'-UUCUCCGAACGUGUCACGUdTT-3', antisense 5'-ACGUGACACGUU CGGAGAAdTT-3'. The siRNAs, supplied as lyophilized, 2'-deprotected desalted duplexes, were suspended in RNase-free buffer (100 mM potassium acetate; 30 mM HEPES-KOH; 2 mM magnesium acetate; pH 7.4) at a concentration of 20 µM. To disrupt higher aggregates that may have formed during lyophilization, the tubes were heated to 90˚C for 1 min and then incubated at 37˚C for 1 h. The siRNAs were aliquoted and stored at -20˚C until required.

2.2.15.2 siRNA delivery and virus infection

S. frugiperda cells were seeded in 24-well tissue culture dishes (1×10^5 cells/well) and transfected with 6-60 pmol of the siRNA, using Oligofectamine™ reagent (Invitrogen) according to the manufacturer's instructions. Briefly, the siRNA was diluted to the appropriate concentration in 40 µl of serum-free Grace's medium, whilst 2 µl of the Oligofectamine™ reagent was diluted in serum-free Grace's medium to a final volume of 7.5 µl. Both solutions were incubated at room temperature for 10 min, then combined and incubated at room temperature for a further 15-20 min to allow formation of oligofectamine-RNA complexes. The cell monolayers were rinsed twice with 500 µl of antibiotic- and serum-free Grace's medium and then overlaid with the oligofectamine-RNA complexes in a final volume of 250 µl. Following incubation at 27˚C for 4 h, the medium was aspirated and replaced with 500 µl of Grace's medium containing 10% (v/v) FCS and antibiotics. At 24 or 48 h post-transfection, the medium was aspirated and the cell monolayers were infected with recombinant Bac-eGFP at a MOI of 2 p.f.u./cell. The tissue culture dishes were incubated at 27˚C in a humid environment and the cells were subsequently assayed for gene knockdown at 24 and 48 h post-infection. Alternatively, *S. frugiperda* cells were seeded in 6-well tissue

culture dishes (1×10^6 cells/well) and transfected with 100 pmol siRNA, using Lipofectamine™2000 reagent (Invitrogen) according to the procedures described by Valdes *et al*. (2003). Following transfection, the *S. frugiperda* cells were incubated for 48 h at 27˚C in a humid environment and then infected with the Bac-eGFP bacmid recombinant at a MOI of 0.05 p.f.u./cell. The tissue culture dishes were incubated at 27˚C and the cells assayed for gene knockdown at 72 h post-infection. *S. frugiperda* cells that had either been mock-transfected or transfected with scrambled (negative control) siRNA were included in all of the above assays.

2.2.15.3 Analysis and quantification of eGFP expression in *S. frugiperda* cells

S. frugiperda cells were observed for the expression of eGFP on a Zeiss Axiovert 200 fluorescent microscope fitted with the no. 10 Zeiss filter set (excitation at 450-490 nm; emission at 515-565 nm). The images were captured for at least three separate microscope fields, using a Nikon DXM1200 digital camera, and analyzed with Nikon ACT-1 v.2.20 software. For fluorometry analysis, the cells were harvested, collected by centrifugation at 5 000 rpm for 10 min and rinsed with 1 × PBS before being suspended in 1 ml of 1 × PBS. The relative fluorescence was determined using a Versafluor™ fluorometer (emission at 515-525 nm and excitation at 485-495 nm; BioRad).

2.3 RESULTS

2.3.1 Construction of pGEM®-T Easy clones containing full-length copies of the AHSV-9 VP7 and enhanced green fluorescent protein (eGFP) genes

2.3.1.1 Construction of plasmid pGEM-VP7

A cDNA copy of the VP7 gene of AHSV-9, flanked by *Bgl*II restriction endonuclease sites, had been cloned previously into the *Bam*HI site of plasmid pBS and the recombinant plasmid was designated pBSS7PCR (Maree, 1998). Although *Bam*HI and *Bgl*II generate compatible 5'-ends, the resultant hybrid site does not constitute a target site for either of the restriction endonucleases. Thus, to facilitate subsequent cloning procedures the full-length VP7 gene was PCR-amplified, using oligonucleotides that were extended at their 5'-ends by additional nucleotides to incorporate unique restriction endonuclease recognition sites.

Using plasmid pBSS7PCR as template DNA, PCR amplification was performed using oligonucleotides VP7-F (containing a *Bgl*II site) and VP7-R (containing a *Kpn*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 (1.150 kb) was observed (Fig 2.3, lane 2). By contrast, no amplification products were observed in the negative control reaction

mixture in which template DNA was omitted (Fig. 2.3, lane 3). Consequently, the amplicon was purified from the agarose gel and ligated into pGEM®-T Easy vector DNA. Following transformation of competent *E. coli* DH5α cells, recombinant transformants with a Gal$^-$ phenotype were selected from X-gal containing indicator plates and cultured in LB broth supplemented with ampicillin. The extracted plasmid DNA was analyzed by agarose gel electrophoresis and by restriction endonuclease digestion.

Plasmid DNA migrating slower than the parental pGEM®-T Easy vector DNA on agarose gels were selected and analyzed for the presence of a cloned insert DNA by using restriction endonucleases of which the recognition sites had been incorporated during the design of the oligonucleotides. The putative recombinant plasmid DNA was therefore digested with both *Bgl*II and *Kpn*I. Following agarose gel electrophoresis, restriction fragments of *ca.* 3.0 and 1.150 kb, respectively, were observed, which is in agreement with the expected size of the pGEM®-T Easy vector DNA (3.0 kb) and insert DNA (1.150 kb) (Fig. 2.3, lane 7). A recombinant clone, designated pGEM-VP7, was selected and the integrity of the cloned insert DNA was verified by sequencing both strands of the cloned insert DNA prior to it being used in further DNA manipulations.

2.3.1.2 Construction of pGEM-eGFP

A recombinant plasmid, designated pGEM-eGFPmut, containing a cloned copy of an eGFP gene was provided by Ms J. Weyer (Department of Microbiology and Plant Pathology, University of Pretoria). Since the recombinant plasmid had not been characterized previously, the nucleotide sequence of the cloned insert DNA was determined by automated sequencing procedures. Comparison of the obtained nucleotide and deduced amino acid sequence to that of an eGFP gene frequently used as reporter in several commercially available vectors (GenBank Acc. No. AAL11719) indicated that the cloned eGFP gene was flanked at its 5'- and 3'-termini by several nucleotides of unknown origin and lacked a termination codon. No other alterations, however, were observed between the respective sequences. Consequently, before the eGFP gene could be used for expression studies in mammalian and insect cells, it was necessary to delete the extra nucleotides at its termini and to incorporate a translational stop codon at its 3'-end. For this purpose, two oligonucleotides were designed, based on the nucleotide sequence obtained, and these were extended at their 5'-ends to incorporate nucleotides specifying unique restriction endonuclease recognition sites to facilitate subsequent cloning procedures. In addition, the 3'-specific oligonucleotide (eGFP-R) was designed to include nucleotides specifying a translational stop codon (TCC) at its 3'-terminus (Table 2.1).

By making use of oligonucleotides eGFP-F (containing a *Bam*HI site) and eGFP-R (containing a *Eco*RI site) and plasmid pGEM-eGFPmut as template DNA, PCR amplification was carried out using the conditions described under Materials and Methods (Section 2.2.3). Following agarose gel electrophoresis of the reaction mixture, an amplicon of *ca.* 720 bp was observed when compared to the size of the DNA molecular weight marker. No amplification products were observed in the negative control reaction mixture in which template DNA was omitted (Fig. 2.4, lanes 2 and 3, respectively). The agarose gel-purified amplicon was subsequently cloned into the pGEM®-T Easy vector, as described in the preceding section. Digestion of the derived recombinant plasmids with both *Bam*HI and *Eco*RI resulted in the excision of a 720-bp DNA fragment, indicating that the amplicon was successfully cloned into the pGEM®-T Easy vector (Fig. 2.4, lane 7). A recombinant clone, designated pGEM-eGFP, was selected and the integrity of the cloned insert DNA, as well as the presence of the newly introduced stop codon, was verified by nucleotide sequencing. The complete nucleotide sequence and deduced amino acid sequence of the cloned eGFP gene is provided in the Appendix to this dissertation.

2.3.2 Construction of recombinant bacmid donor plasmids containing the AHSV-9 VP7, enhanced green fluorescent protein (eGFP) and chimeric VP7-eGFP genes

To enable cloning of the eGFP gene into the bacmid donor plasmid pFastBac1™, both the recombinant pGEM-eGFP and pFastBac1™ plasmid DNA were digested with *Bam*HI and *Eco*RI. The excised eGFP gene and digested vector DNA were purified from an agarose gel and ligated overnight. Similarly, the AHSV-9 VP7 gene was excised from recombinant plasmid pGEM-VP7 by digestion with both *Bgl*II and *Kpn*I and then ligated to pFastBac1™, which had been digested with both *Bam*HI and *Kpn*I. Following transformation of competent *E. coli* DH5α cells, a number of transformants were selected randomly and the extracted plasmid DNA was subsequently characterized by restriction enzyme digestion. Recombinant plasmids from which either a 720-bp eGFP-specific (Fig. 2.5B, lane 7) or 1.050-kb VP7-specific (Fig. 2.5B, lane 9) insert DNA was excised, were selected for further use and designated pFB-eGFP and pFB-VP7, respectively.

Although a chimeric VP7-eGFP gene had been constructed in the mammalian expression vector pCMV-Script® (Chapter 3), it was not possible to simply excise and reclone the chimeric gene into the pFastBac1™ donor plasmid. This was as a consequence of the cloning strategy used, since the hybrid site, resulting from ligation of the compatible *Bam*HI and *Bgl*II cohesive termini, does not constitute a target site for either of these restriction enzymes. Therefore, the chimeric gene was obtained by PCR amplification using the recombinant pCMV-VP7-eGFP plasmid DNA as

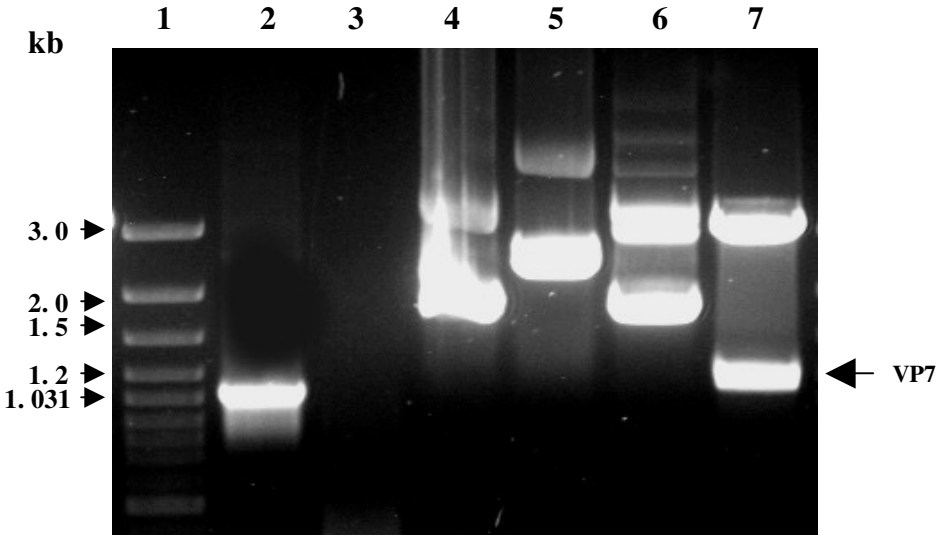

Fig. 2.3 **Agarose gel electrophoretic analysis of the recombinant plasmid pGEM-VP7. Lane 1, DNA molecular weight marker; lane 2, amplicon obtained by PCR amplification using pBSS7PCR plasmid DNA as template and oligonucleotides VP7-F and VP7-R; lane 3, negative control PCR reaction mixture lacking template DNA; lane 4, uncut parental pGEM®-T Easy vector DNA; lane 5, uncut recombinant plasmid pGEM-VP7; lane 6, pGEM®-T Easy vector DNA digested with both *Bgl*II and *Kpn*I (vector lacks both restriction endonuclease sites); lane 7, recombinant plasmid pGEM-VP7 digested with both *Bgl*II and *Kpn*I. The sizes of the DNA molecular weight marker, GeneRuler™ 100-bp DNA Ladder Plus (Fermentas), are indicated to the left of the figure.**

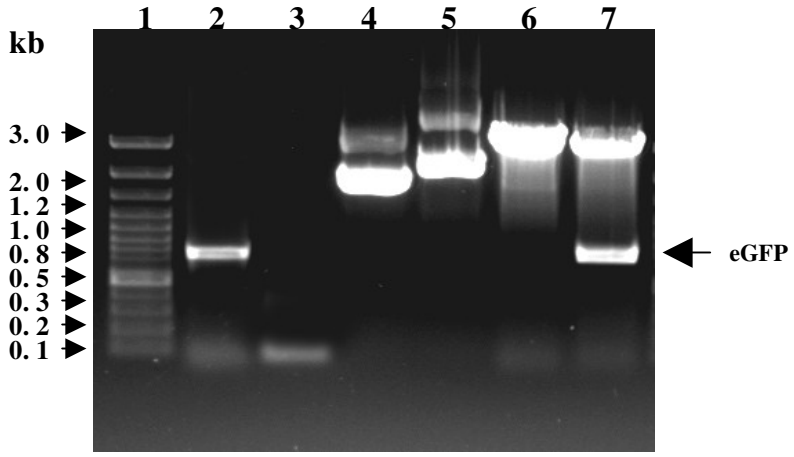

Fig. 2.4 **Agarose gel electrophoretic analysis of the recombinant plasmid pGEM-eGFP. Lane 1, DNA molecular weight marker; lane 2, amplicon obtained by PCR amplification using pGEM-eGFPmut plasmid DNA as template and oligonucleotides eGFP-F and eGFP-R; lane 3, negative control PCR reaction mixture lacking template DNA; lane 4, uncut parental pGEM®-T Easy vector DNA; lane 5, uncut recombinant plasmid pGEM-eGFP; lane 6, pGEM®-T Easy vector DNA digested with both *Bam*HI and *Eco*RI; lane 7, recombinant plasmid pGEM-eGFP digested with both *Bam*HI and *Eco*RI. The sizes of the DNA molecular weight marker, GeneRuler™ 100-bp DNA Ladder Plus (Fermentas), are indicated to the left of the figure.**

template, together with oligonucleotides VP7-F (containing a *Bgl*II site) and eGFP-RXhoI (containing a *Xho*I site). Following PCR, the 1.773-kb amplicon was cloned into pGEM®-T Easy to generate pGEM-VP7-eGFP. The integrity of the cloned insert DNA was verified by nucleotide sequencing of the full-length VP7-eGFP gene prior to it being recovered by digestion with both *Bgl*II and *Xho*I and then cloned into pFastBac1™, which had been prepared by digestion with both *Bam*HI and *Xho*I, to generate pFB-VP7-eGFP. The recombinant bacmid donor plasmid was subsequently characterized by digestion with both *Bam*HI, which cuts 75 nucleotides from the 5'-end of the VP7 gene, and *Hind*III, which cuts at the 5'-end of the eGFP gene and once in the multiple cloning site of the vector. Following agarose gel electrophoresis of the reaction mixture, two DNA fragments corresponding in size with the VP7 gene (1.050 kb) and eGFP gene (720 bp) could be observed (Fig. 2.5B, lane 11), thus confirming that the chimeric VP7-eGFP gene had been cloned successfully.

2.3.3 Engineering of recombinant bacmids

Bacmid DNA replicates as a large plasmid in *E. coli* DH10Bac™ cells and confers resistance to kanamycin, as well as complements a *lacZ*-deletion present on the *E. coli* chromosome to form colonies that are blue in the presence of X-gal. During the site-specific transposition of the recombinant donor plasmid, a mini-Tn7 cassette is inserted from the donor plasmid into the mini-*att*Tn7 attachment site on the bacmid DNA, thereby disrupting expression of the LacZα peptide. Consequently, colonies containing the recombinant bacmid display a white phenotype and can be easily distinguished from blue colonies that harbour the unaltered bacmid when plated in the presence of X-gal and IPTG. The transposition functions are provided *in trans* by a helper plasmid (pMON7124) that encodes the transposase and confers resistance to tetracycline (Luckow *et al*., 1993).

Recombinant bacmid DNA was therefore engineered by transforming competent *E. coli* DH10Bac™ cells with the recombinant plasmids pFB-VP7, pFB-eGFP and pFB-VP7-eGFP, and selecting for recombinant bacmid DNA by plating of the transformed cells onto a selective medium. The high-molecular-weight recombinant DNA was subsequently extracted from transformed *E. coli* DH10Bac™ cells and successful transposition of the respective genes into the bacmid DNA was investigated by PCR analyses. The oligonucleotides used in this assay were the pHed-F and universal pUC/M13-R oligonucleotides. Whereas the pHed-F oligonucleotide anneals to the polyhedrin promoter of the transposed vector DNA, the pUC/M13-R oligonucleotide anneals to the 3'-end of the mini-*att*Tn7 site within the *lacZ*α gene of the bacmid DNA. Alternatively, gene-

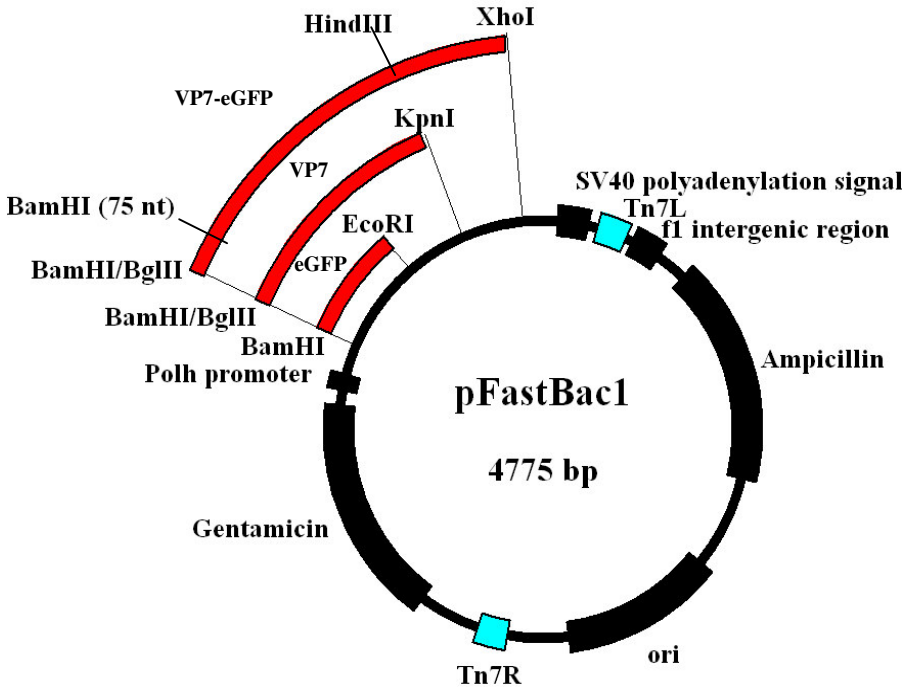

Fig. 2.5A **Plasmid map of the recombinant plasmids pFB-eGFP, pFB-VP7 and pFB-VP7-eGFP harbouring the eGFP (720 bp), VP7 (1.050 kb) and VP7-eGFP (1.773 kb) genes, respectively.**

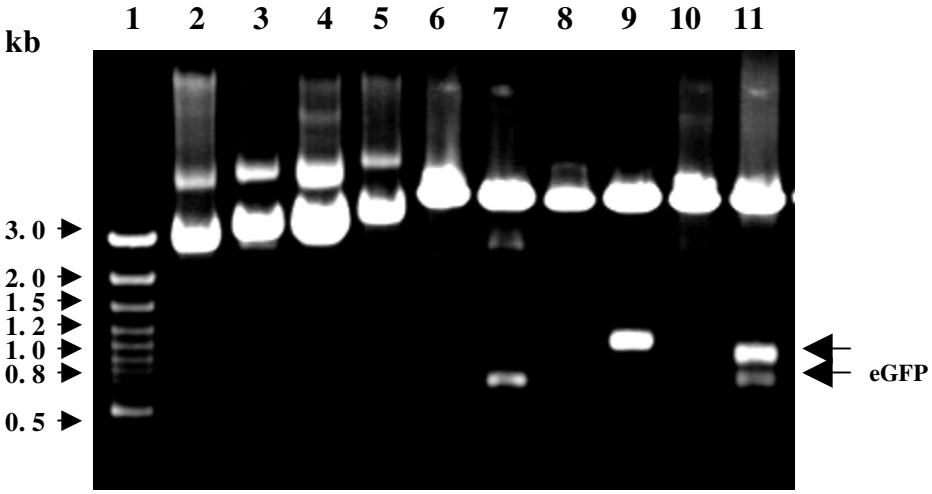

Fig. 2.5B **Agarose gel electrophoretic analysis of the recombinant pFastBac1™ donor plasmids pFB-eGFP, pFB-VP7 and pFB-VP7-eGFP. Lane 1, DNA molecular weight marker; lane 2, uncut parental pFastBac1™ vector DNA; lane 3, uncut recombinant plasmid pFB-eGFP; lane 4, uncut recombinant plasmid pFB-VP7; lane 5, uncut recombinant plasmid pFB-VP7-eGFP; lane 6, parental pFastBac1™ vector DNA digested with both *Bam*HI and *Eco*RI; lane 7, pFB-eGFP vector DNA digested with *Bam*HI and *Eco*RI; lane 8, parental pFastBac1™ vector DNA digested with both *Bam*HI and *Kpn*I; lane 9, pFB-VP7 vector DNA digested with both *Bam*HI and *Kpn*I; lane 10, parental pFastBac1™ vector DNA digested with both *Bam*HI and *Hind*III; lane 11, recombinant plasmid pFB-VP7-eGFP DNA digested with both *Bam*HI and *Hind*III. The sizes of the DNA molecular weight marker, GeneRuler™ 100-bp DNA Ladder Plus (Fermentas), are indicated to the left of the figure.**

specific oligonucleotides were used in combination with the universal pUC/M13-R oligonucleotide (Fig. 2.6A).

By making use of recombinant bacmid DNA transposed with the pFB-eGFP donor plasmid as template for the PCR, a single band of 720 bp was obtained using the gene-specific oligonucleotides eGFP-F and eGFP-R (Fig. 2.6B, lane 5), whilst a band of *ca.* 1.323 kb was obtained using the eGFP-F and pUC/M13-R oligonucleotides (Fig. 2.6B, lane 6). This band corresponded with the size of the eGFP gene together with *ca.* 600 bp of the mini-Tn7 cassette. By contrast, when wild-type bacmid DNA was used as template in the PCR reactions, no amplification products were observed (Fig. 2.6B, lanes 2 and 3, respectively). The recombinant bacmid was designated Bac-eGFP and used in all subsequent experiments.

By making use of recombinant bacmid DNA transposed with either the pFB-VP7 or pFB-VP7-eGFP donor plasmids as templates for PCR, together with the pHed-F and universal pUC/M13-R oligonucleotides, bands of *ca.* 1.650 kb (Fig. 2.6C, lanes 3-8) and 2.373 kb (Fig. 2.6D, lanes 2-6) were obtained, respectively. These corresponded with the size of the VP7 gene (1.050 kb) or VP7-eGFP chimeric gene (1.773 kb), together with the size of the mini-Tn7 cassette (458 bp) and bacmid DNA flanking the mini-*att*Tn7 site (145 bp). By contrast, no amplification products were observed when wild-type bacmid DNA was used as templates in the PCR reactions or in control reactions in which template DNA was omitted. Recombinant bacmids were selected, designated Bac-VP7 and Bac-VP7-eGFP, respectively, and used in all subsequent experiments.

2.3.4 Analyses of proteins synthesized in recombinant bacmid-infected *S. frugiperda* cells

The recombinant bacmid DNA was isolated from *E. coli* DH10Bac™ cultures and subsequently transfected into *S. frugiperda* cells. Progeny viruses, resulting from each transfection, were propagated in monolayer cultures and the titre of the recombinant virus stocks was determined by plaque assays. To determine whether the eGFP, AHSV-9 VP7 and VP7-eGFP proteins were expressed in *S. frugiperda* cells by the respective bacmid recombinants, monolayers of *S. frugiperda* cells were mock-infected and infected at a MOI of 3-8 with either the wild-type or different bacmid recombinants. At 72 h post-infection, the monolayers were examined by epifluorescence microscopy, after which the cells were harvested and the cell lysates were analysed by SDS-PAGE and immunoblot analyses.

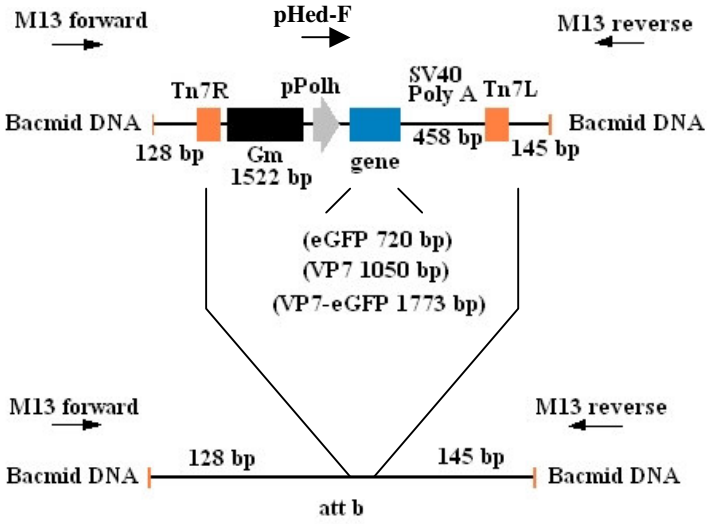

Fig. 2.6A **Schematic representation of the bacmid transposition region showing the annealing positions of the universal pUC/M13 forward and reverse oligonucleotides, as well as that of the pHed-F oligonucleotide.**

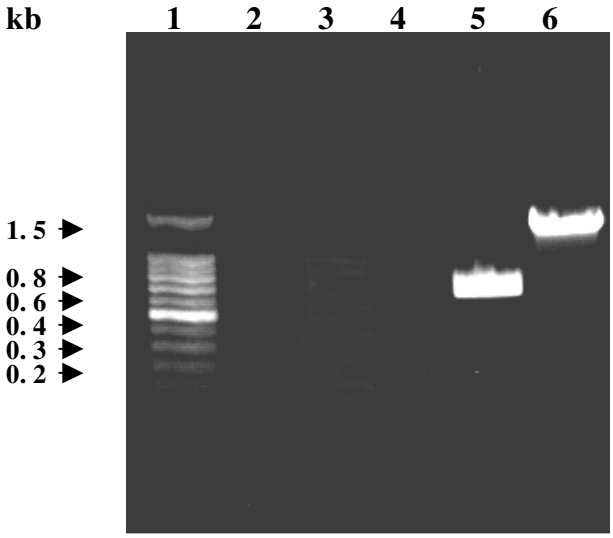

Fig. 2.6B **Agarose gel electrophoretic analysis of the recombinant Bac-eGFP DNA. Lane 1, DNA molecular weight marker; lane 2, wild-type bacmid DNA used as template in PCR amplification and oligonucleotides eGFP-F and eGFP-R; lane 3, wild-type bacmid DNA used as template in PCR amplification and primers eGFP-F and pUC/M13-R; lane 4, negative control PCR reaction mixture lacking template DNA; lane 5, amplicon obtained by PCR amplification using recombinant bacmid-eGFP DNA as template and oligonucleotides eGFP-F and eGFP-R; lane 6, amplicon obtained by PCR amplification using recombinant bacmid-eGFP DNA as template and oligonucleotides eGFP-F and pUC/M13-R. The sizes of the DNA molecular weight marker, 100-bp DNA ladder (Promega), are indicated to the left of the figure.**

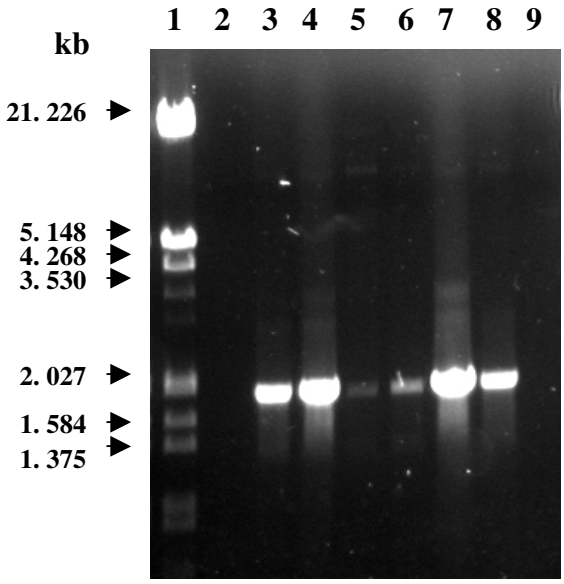

Fig. 2.6C **Agarose gel electrophoretic analysis of the recombinant Bac-VP7 DNA. Wild-type bacmid DNA (lane 2) or recombinant bacmid VP7 DNA (lanes 3-8) was used as template in PCR amplification with oligonucleotides pHed-F and pUC/M13-R. A negative control PCR reaction mixture lacking template DNA (lane 9) was also included in the analysis. The sizes of the molecular weight marker (lane 1), phage λ DNA (Roche Diagnostics) digested with *Hind*III and *Eco*RI, are indicated to the left of the figure.**

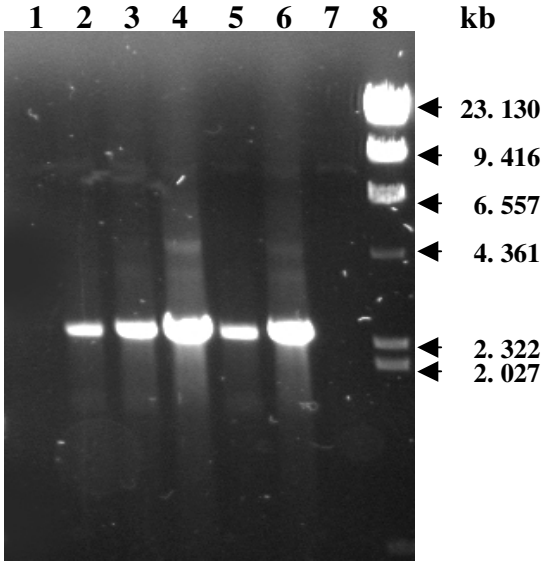

Fig. 2.6D **Agarose gel electrophoretic analysis of the recombinant Bac-VP7-eGFP DNA. Wild-type bacmid DNA (lane 7) or recombinant bacmid VP7-eGFP DNA (lanes 2-6) was used as template in PCR amplification with oligonucleotides pHed-F and pUC/M13-R. A negative control PCR reaction mixture lacking template DNA (lane 1) was also included in the analysis. The sizes of the molecular weight marker (lane 8), phage λ DNA (Roche Diagnostics) digested with *Hind*III, are indicated to the right of the figure.**

2.3.4.1 Epifluorescence microscopy of cell monolayers

Examination of the mock-infected and recombinant bacmid-infected *S. frugiperda* cell monolayers by epifluorescence microscopy (Fig. 2.7) indicated that cells infected with the Bac-eGFP and Bac-VP7-eGFP bacmid recombinants fluoresced brightly. As expected, no fluorescence was observed in control mock-infected cells or in cells infected with either the wild-type bacmid or Bac-VP7 recombinant bacmid. Due to the absence of background- and autofluorescence in the latter cell monolayers, it was concluded that the fluorescence observed in Bac-eGFP and Bac-VP7-eGFP infected cells was due to successful expression of the eGFP and VP7-eGFP proteins. Notably, the fluorescence pattern appeared to differ between these infected cells. Whereas cells infected with Bac-eGFP displayed a homogenous fluorescent pattern (Fig. 2.7D), the fluorescent pattern of cells infected with Bac-VP7-eGFP appeared to be more punctuated and discreet brightly fluorescent loci were readily visible in the cytoplasm of the infected cells (Fig. 2.7F). This may be representative of VP7-eGFP protein that had aggregated into crystalline particles (also see Fig. 2.10) during the late stage of virus infection (72 h).

2.3.4.2 SDS-PAGE and immunoblot analyses

Analysis of the Coomassie blue-stained gel (Fig. 2.8A) indicated the presence of a unique over-expressed protein in each of the lysates prepared from cell monolayers infected with the Bac-VP7 (lane 4), Bac-VP7-eGFP (lane 5) or Bac-eGFP (lane 6) bacmid recombinants. The molecular mass of the unique proteins was in agreement with the predicted molecular mass of the structural protein VP7 of AHSV-9 (38 kDa), the VP7-eGFP chimeric protein (65 kDa) and the eGFP protein (27 kDa). No similar proteins were observed in lysates prepared from mock-infected or wild-type bacmid-infected cells. Subsequent immunoblot analyses of the respective cell lysates indicated that the uniquely expressed proteins were recognized by an AHSV-9-specific antiserum (Fig. 2.8B) or polyclonal anti-eGFP antibody (Fig. 2.8C). Although the AHSV-9 antiserum reacted with the 65-kDa VP7-eGFP chimeric protein and the 38-kDa VP7 protein, proteins migrating slightly faster than the respective proteins were also seen to react with the antiserum (Fig. 2.8B, lanes 4 and 5, respectively). Since no cross-reaction of the antiserum with proteins from mock- and wild-type bacmid-infected *S. frugiperda* cells were observed, it was concluded that these smaller proteins represented degraded derivatives of the respective proteins. By contrast, the anti-eGFP antibody reacted specifically with the 27-kDa eGFP protein, in addition to the VP7-eGFP chimeric protein (Fig. 2.8C, lanes 6 and 5, respectively). From these results it could thus be concluded that the VP7, eGFP and VP7-eGFP proteins were expressed successfully in *S. frugiperda* cells by means of the constructed bacmid recombinants.

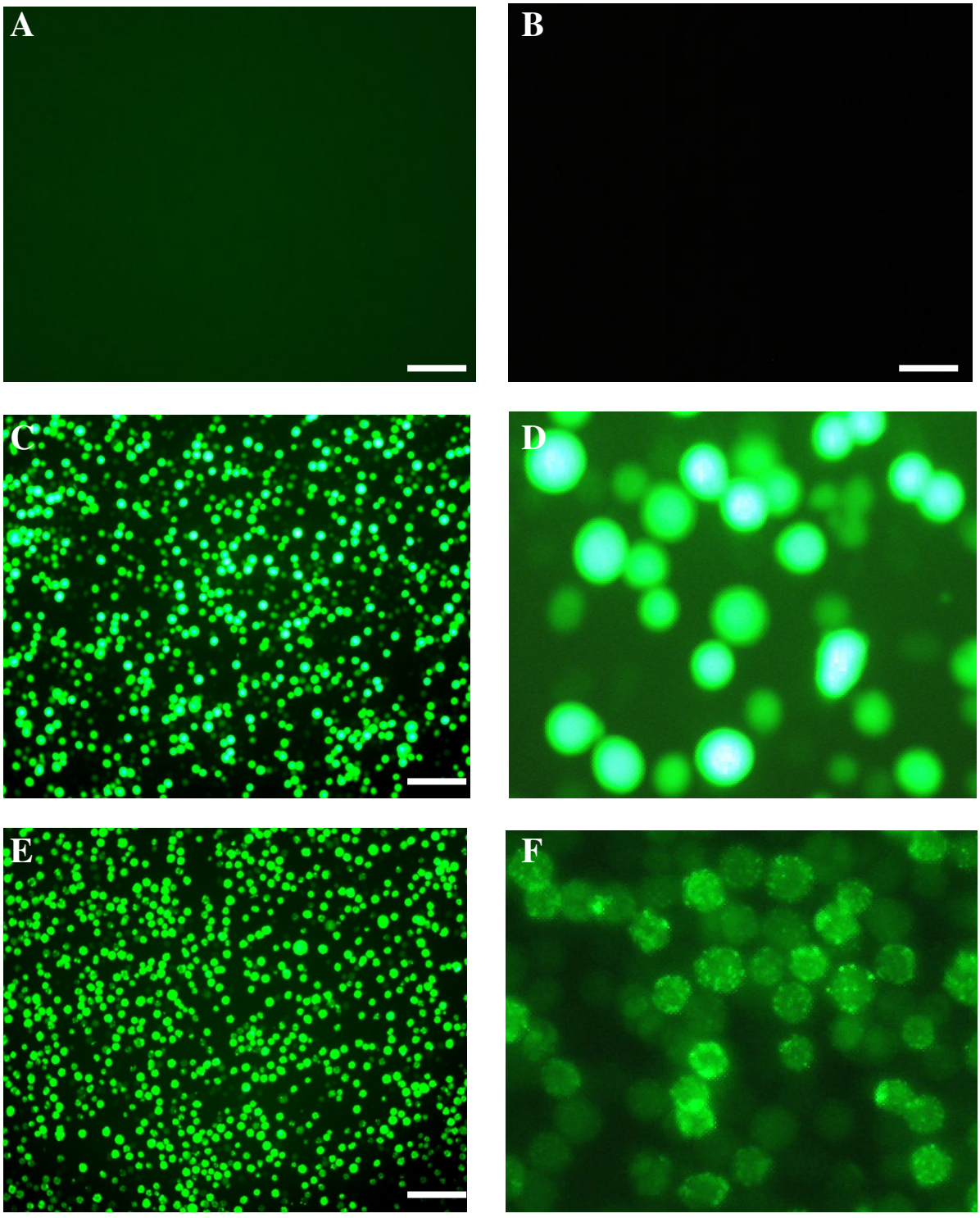

Fig. 2.7 **Epifluorescence microscopy of recombinant bacmid-infected *S. frugiperda* cells. *S. frugiperda* cells were mock-infected (A) and infected with the wild-type bacmid (B) or recombinant bacmids Bac-eGFP (C and D) and Bac-VP7-eGFP (E and F), and analyzed for protein expression at 72 h post-infection using a Zeiss Axiovert fluorescent microscope. Micrographs D and F are enlarged to indicate the difference in fluorescent protein distribution between *S. frugiperda* cells infected with Bac-eGFP (D) and Bac-VP7-eGFP (F). Bar = 100 µm.**

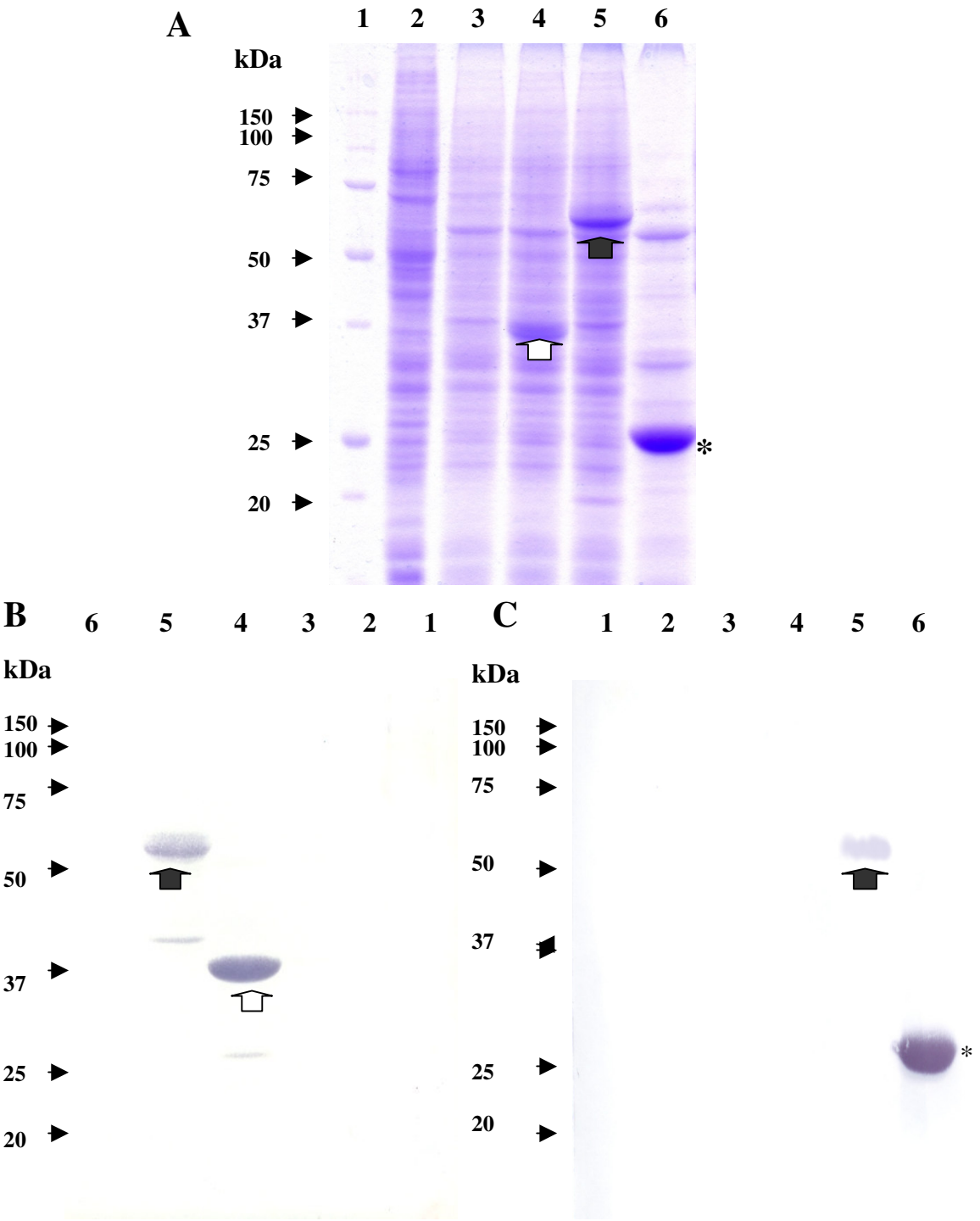

Fig. 2.8 **SDS-PAGE and immunoblot analyses of cell lysates from recombinant bacmid-infected *S. frugiperda* cells. The cell lysates were resolved by SDS-PAGE (A) and the duplicate, unstained SDS-PAGE gels were electroblotted onto nitrocellulose membranes and subjected to immunoblot analysis using AHSV-9 antiserum (B) or polyclonal anti-eGFP antibodies (C). Lanes 1, Protein molecular weight marker; lanes 2, mock-infected cells; lanes 3, cells infected with wild-type bacmid; lanes 4, cells infected with Bac-VP7; lanes 5, cells infected with Bac-VP7-eGFP; lanes 6, cells infected with Bac-eGFP. The sizes of the molecular weight marker (BioRad) are indicated to the left of the figures. In all figures, VP7 and VP7-eGFP are indicated with open and closed arrows, respectively, whilst eGFP is indicated by an asterisk.**

2.3.5 Characterization of the bacmid-expressed AHSV VP7 and VP7-eGFP proteins

It has been reported previously that VP7 of AHSV forms trimers and that overexpression of the protein in *S. frugiperda* cells by means of baculovirus recombinants results in the protein aggregating into hexagonal crystalline particles (Chuma *et al*., 1992; Burroughs *et al*., 1994). Thus, to investigate whether the in-frame addition of eGFP to the C-terminus of VP7 may affect its ability to assemble into similar particles, cytoplasmic extracts of recombinant bacmid-infected cells were prepared and, following isopycnic centrifugation and fractionation from the bottom of each gradient, the gradients were compared by SDS-PAGE.

The results obtained (Fig. 2.9) indicated that most of the VP7 and VP7-eGFP proteins reached equilibrium within fraction 5, indicating that there is not a major change in particle density. Notably, a low amount of monomeric VP7, but not VP7-eGFP, was detectable at the top of the gradient. The slight difference in sedimentation of the two proteins did not depend on the level of expression in the recombinant bacmid-infected cells or on the amount of protein loaded onto the gradients. These results therefore indicate that the addition of eGFP to the C-terminus of AHSV-9 VP7 altered its sedimentation profile only slightly, resulting in the formation of complexes with no monomeric VP7-eGFP being present. Furthermore, fluorescence analysis of each VP7-eGFP fraction by means of fluorometry correlated with the SDS-PAGE analysis of protein content in each fraction.

The sucrose gradient-purified VP7 and chimeric VP7-eGFP proteins were subsequently examined by scanning electron microscopy (SEM). The results, presented in Fig. 2.10, revealed distinct morphological differences between the particles formed by VP7 and those formed by VP7-eGFP. Whereas the VP7 protein aggregated into flat, smooth hexagonal particles with a diameter of 6-8 µm, the particles formed by the VP7-eGFP chimeric protein were larger (10-12 µm) and displayed a rough, circular appearance. No discernible hexagonal crystalline particles were observed in these preparations. A compound appearing as amorphous foamy white material, present on both the protein surface and filter, was detected especially where clusters of the VP7-eGFP particles occurred on the grid. This may represent cellular proteins, since the proteins were not purified to homogeneity prior to SEM analysis. Nevertheless, the results obtained indicated that the C-terminal addition of eGFP affected adversely the ability of the baculovirus-expressed VP7 proteins to form hexagonal particles.

A

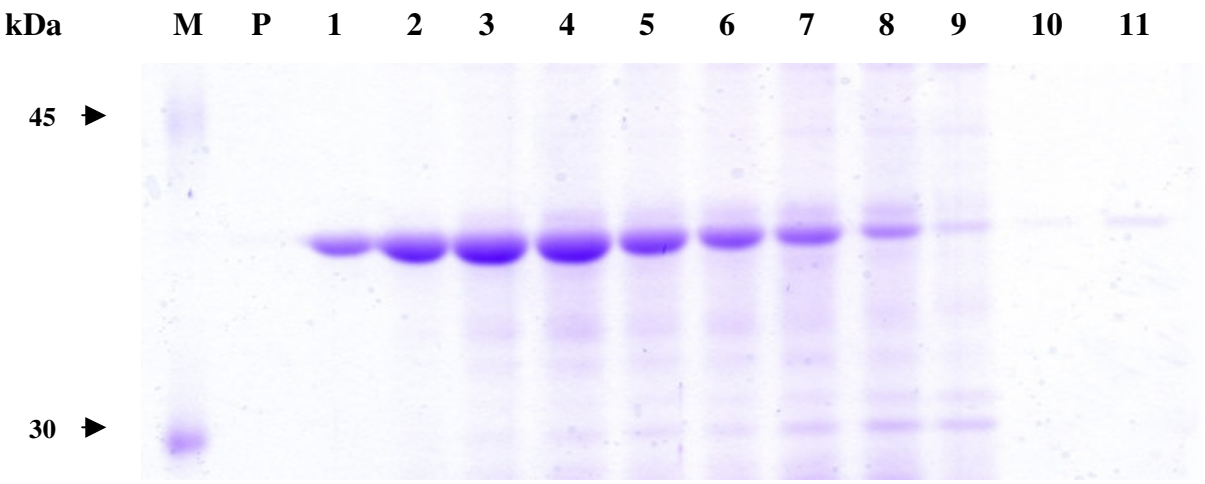

Fig. 2.9A **Sedimentation analysis of the cytoplasmic extract from cells infected with Bac-VP7. Fractions were collected from the bottom (lane 1) to the top (lane 11) of each gradient, following sucrose density centrifugation at 40 000 rpm for 18 h and analyzed by SDS-PAGE. The pellet fraction is indicated as P. The sizes of the protein molecular weight marker (lane M) are indicated to the left of the figure.**

B

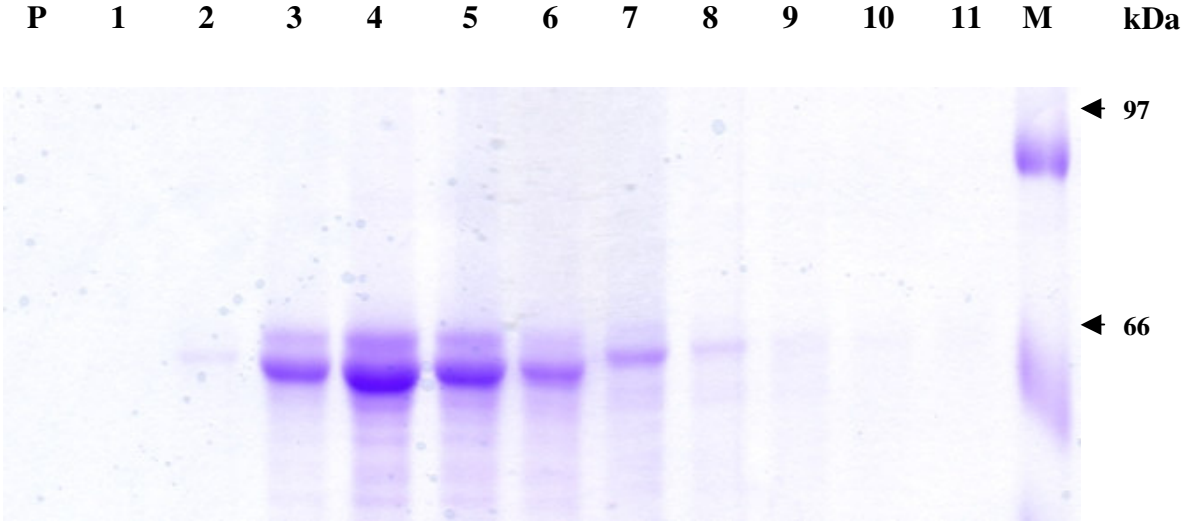

Fig. 2.9B **Sedimentation analysis of the cytoplasmic extract prepared from cells infected with Bac-VP7-eGFP. Fractions were collected from the bottom (lane 1) to the top (lane 11) of each gradient, following sucrose density centrifugation at 40 000 rpm for 18 h and analyzed by SDS-PAGE. The pellet fraction is indicated as P. The sizes of the protein molecular weight marker (lane M) are indicated to the right of the figure.**

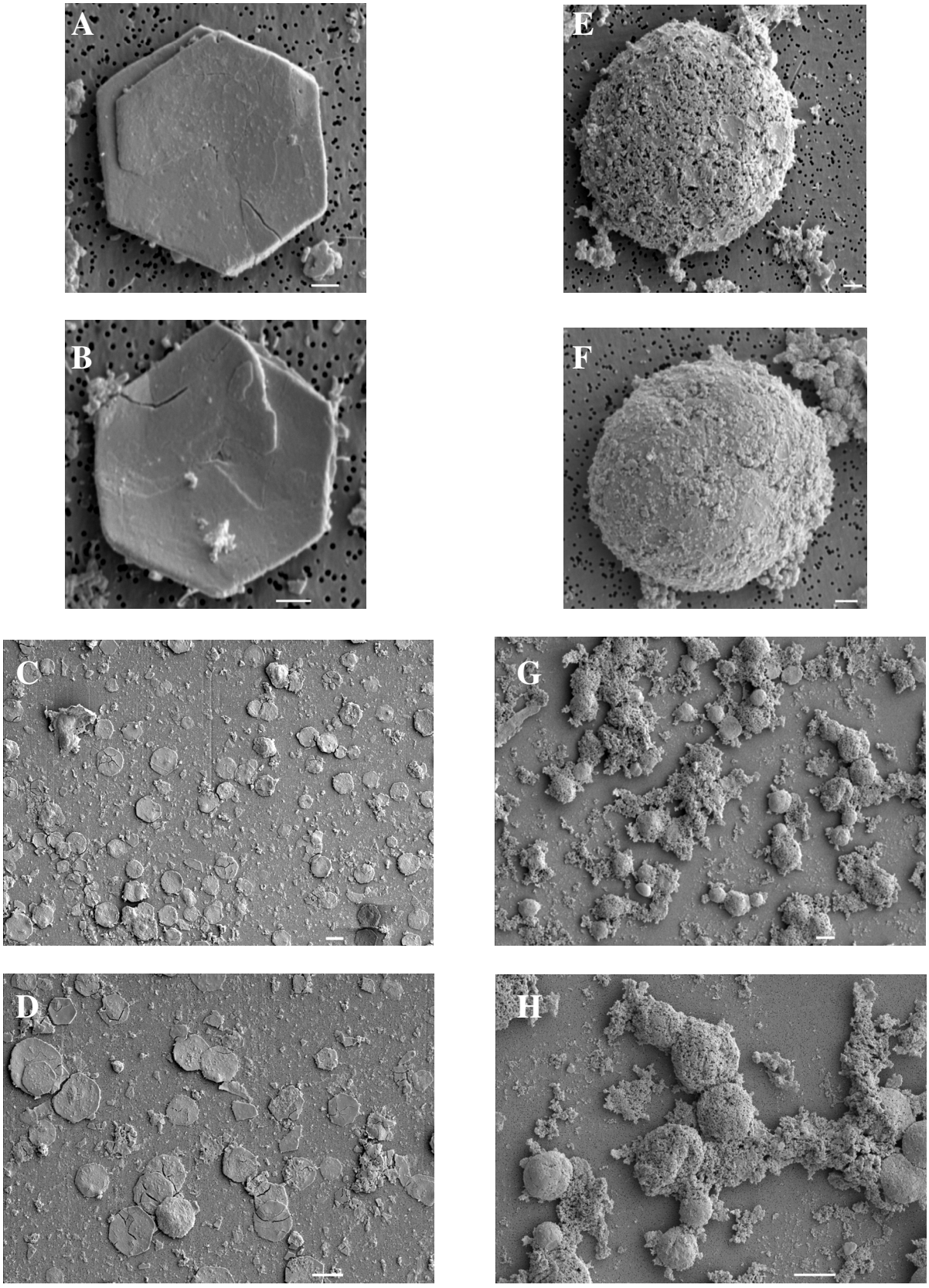

Fig. 2.10 **Scanning electron micrographs of sucrose gradient-purified wild-type AHSV-9 VP7 (A-D) and VP7-eGFP (E-H) particles. The bar in A, B, E and F represents 1 µm, and that in C, D, G and H represents 10 µm.**

2.3.6 Inhibition of eGFP expression by siRNA in *S. frugiperda* cells

Baculovirus recombinants are frequently used by members of our research group to express and study the function of orbivirus structural and nonstructural proteins, and have also been used to study the formation of AHSV core- and virus-like particles (Maree and Huismans, 1997; Maree *et al.*, 1998; van Staden *et al.*, 1998). With a view towards facilitating such studies, the possibility of silencing a transgene expressed by a bacmid recombinant in *S. frugiperda* cells was subsequently explored.

Towards establishing optimal assay conditions, *S. frugiperda* cells were transfected with different concentrations of an eGFP-specific siRNA (siGFP-22) and subsequently infected with Bac-eGFP at different multiplicities of infection. Based on the results obtained, *S. frugiperda* cells were consequently transfected in duplicate with 50 pmol of siGFP-22 and then infected with Bac-eGFP at 24 or 48 h post-transfection at a MOI of 2. The cell monolayers were examined by fluorescence microscopy at 24 and 48 h post-infection and the eGFP fluorescence was quantified by fluorometry. The results obtained (Fig. 2.11A) indicated that transfection of the *S. frugiperda* cells with siGFP-22 48 h prior to infection with the recombinant bacmid, suppressed eGFP expression by 26% at 24 h post-infection and by 28% at 48 h post-infection. However, infection of the siGFP-22-transfected *S. frugiperda* cells at 24 h post-transfection suppressed eGFP expression by 75% at 24 h post-infection, but decreased to 54% at 48 h post-infection. To improve on this low efficiency in inhibition of transgene expression, an approach described by Valdes *et al.* (2003) was followed, which reportedly results in a 91% inhibition of eGFP expression in *S. frugiperda* cells. Thus, *S. frugiperda* cells were transfected with 100 pmol of siGFP-22 and then infected with Bac-eGFP at 48 h post-transfection at a MOI of 0.05. The cell monolayers were examined by fluorescence microscopy at 72 h post-infection and the eGFP fluorescence was quantified by fluorometry. The results indicated that eGFP expression was suppressed by 53% and appeared to be gene-specific, since scrambled (negative control) siRNA had no effect on eGFP expression (Fig. 2.11B).

Despite different RNAi assay conditions, the extent to which eGFP expression could be inhibited remained inefficient. It should, however, be noted that bacmid-infected cells will lyse eventually, thereby abrogating the RNAi effect and complicating interpretation of the results. The lack of complete or near complete inhibition of transgene expression precluded application of the developed RNAi assay to study inhibition of AHSV VP7 or chimeric VP7-eGFP gene expression.

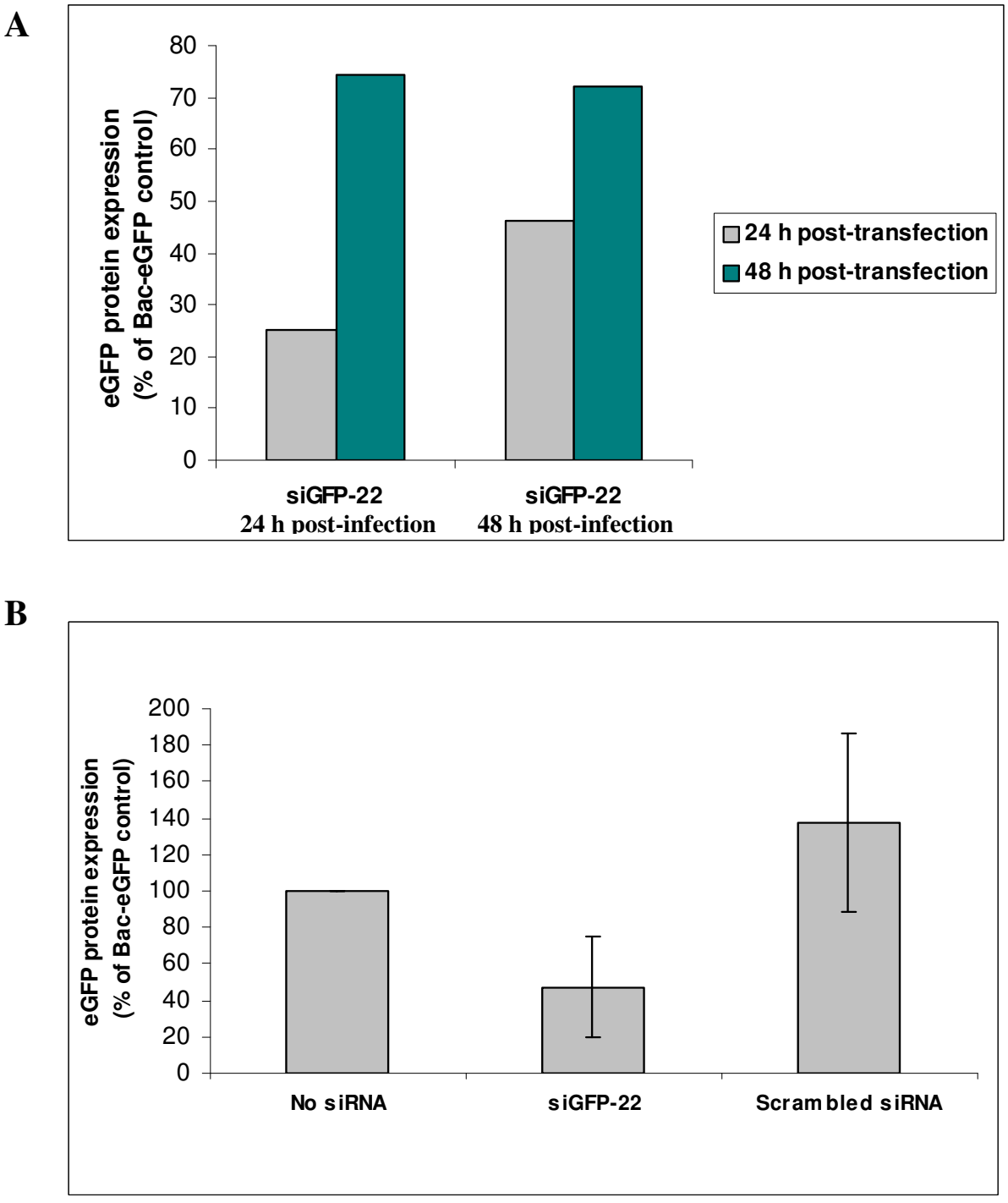

Fig. 2.11 **Inhibition of eGFP protein expression in *S. frugiperda* cells. (A) *S. frugiperda* cells were transfected with 50 pmol siGFP-22 and then infected with Bac-eGFP at 24 or 48 h post-transfection at a MOI of 2. Cells were analyzed for fluorescence 24 and 48 h post-infection. (B) *S. frugiperda* cells were transfected with 100 pmol siRNA and fluorescence was measured 72 h after Bac-eGFP infection at a MOI of 0.05. siGFP-22, cells transfected with eGFP-specific siRNA; Scrambled siRNA, cells transfected with scrambled control siRNA. Bars show mean ± SD for two independent experiments.**

2.4 DISCUSSION

RNA interference (RNAi) is an evolutionarily conserved gene silencing mechanism that is emerging as a powerful and efficient tool to determine the function of genes and its encoded proteins (Cheng *et al*., 2003; Shin *et al*., 2003; Shen, 2004; Lu, 2004). Both dsRNAs and siRNAs have been used successfully to specifically silence exogenous and endogenous genes in cultured mammalian and insect cells (Clemens *et al*., 2000; Caplen *et al*., 2001; Elbashir *et al*., 2001a; Valdes *et al*., 2003; Agrawal *et al*., 2004), and in a wide range of other eukaryotic organisms (Agrawal *et al*., 2003). Several reports have also demonstrated successful inhibition of replication of a wide variety of viruses in cultured cells (Haasnoot *et al*., 2003; Tan and Yin, 2004), and these results have generated optimism that RNAi may hold therapeutic potential for both human (McCaffrey *et al.*, 2003; Tompkins *et al.*, 2004) and animal (Chen *et al*., 2004; Liu *et al.*, 2005) viral diseases.

Based on our interest in elucidating the function of AHSV proteins by expression of these proteins in the bacmid/insect cell expression system and on the successes reported regarding siRNA-mediated silencing of baculovirus genes in insect cell culture (Means *et al*., 2003; Valdes *et al*., 2003; Agrawal *et al*., 2004; Ikeda *et al*., 2004), an attempt was made to develop an RNAi strategy whereby transgene expression in *S. frugiperda* insect cells could be inhibited by using siRNA corresponding to the transgene. Towards developing such an RNAi assay, recombinant bacmids were engineered expressing VP7 of AHSV-9, an enhanced green fluorescent protein (eGFP) or a chimeric VP7-eGFP protein. It was reasoned that the eGFP reporter gene, which encodes a GFP variant that fluoresces 35 times brighter than wild-type protein (Cormack *et al*., 1996), would provide a simple and impartial model for assessing the efficacy of gene silencing by analysis at the protein level visually and quantitatively by fluorometry.

The recombinant bacmids that were generated harboured a cloned copy of the VP7, eGFP and chimeric VP7-eGFP genes under transcriptional control of the strong polyhedrin promoter, which directs high-level protein synthesis during the late stages of baculovirus infection (Matsuura *et al.*, 1987; Gombart *et al.*, 1989). Using this promoter, the expression of foreign proteins can represent 20 to 50% of the total proteins of the infected insect cell (Rohel *et al.*, 1983; Matsuura *et al.*, 1987). SDS-PAGE analysis of cell lysates prepared from the recombinant bacmid-infected *S. frugiperda* cells indicated the presence of an overexpressed unique protein in each of the lysates that was absent from the control mock-infected and wild-type bacmid-infected cell lysates. The identity of the respective proteins was subsequently confirmed by immunoblot analysis and the AHSV-specific

antiserum reacted specifically with the AHSV VP7 (38 kDa) and chimeric VP7-eGFP (65 kDa) proteins, whilst a polyclonal anti-eGFP antibody reacted specifically with the eGFP (27 kDa) and chimeric VP7-eGFP proteins. Notably, the AHSV-specific serum reacted with a protein migrating faster than those corresponding to the full-length VP7 and chimeric V7-eGFP proteins (Fig. 2.8B). Since *S. frugiperda* cells infected with the recombinant bacmid expressing the chimeric VP7-eGFP protein fluoresced brightly, these results suggest that the observed lower molecular mass proteins may represent amino (N)-terminus truncated versions of the full-length polypeptides. This is furthermore supported by the observation that no similar protein was detected in an immunoblot of VP7-eGFP using an anti-eGFP antibody (Fig. 2.8C). The origin of these truncated proteins, however, was not investigated further in this study.

It has been reported previously that AHSV VP7 molecules form trimers and that overexpression of the protein in *S. frugiperda* cells (Chuma *et al*., 1992) and in virus-infected BHK-21 cells (Burroughs *et al*., 1994) results in it spontaneously assembling into large hexagonally shaped, crystalline particles. This characteristic of AHSV VP7 is currently being exploited towards the development of a particulate vaccine delivery system (Prof H. Huismans, Department of Genetics, University of Pretoria). Consequently, the chimeric VP7-eGFP protein was investigated for its ability to assemble into hexagonal particles, similar to those observed for VP7 in infected *S. frugiperda* cells. To investigate, cytoplasmic extracts of recombinant bacmid-infected cells were prepared and analyzed by sedimentation analysis in sucrose gradients. Subsequent SEM examination of the protein-containing fractions indicated that, in contrast to the VP7 protein, which aggregated into hexagonal crystalline particles, the VP7-eGFP particulate structures displayed a circular, almost “ball-like” appearance. Since VP7 trimer-trimer interactions most likely involve the C-termini of VP7 molecules (Basak *et al.*, 1996) and based on reports indicating that the last 16 residues at the C-terminus of VP7 ties the trimers together during capsid and crystal formation (Monastyrskaya *et al*., 1997), it is possible that the fusion of eGFP to the C-terminus of VP7 resulted in distortion of the VP7 crystalline particle, possibly by interfering with protein folding and/or trimerization. Furthermore, it has been shown that a single point mutation in BTV-10 VP7 prevents the formation of core-like particle (Le Blois and Roy, 1993).

To develop an RNAi approach for transgene inhibition in recombinant bacmid-infected *S. frugiperda* cells and to assess its feasibility, eGFP was used as the target transgene and the ability of cognate siGFP-22 to silence the expression of eGFP was examined. Following optimization of several parameters (amongst other; concentration of siGFP-22 used, cell density, MOI of virus infection, differences in the time between siRNA transfection and virus infection, as well as and

combinations thereof), best inhibition of eGFP expression was observed when 50 pmol of siGFP-22 was transfected into the *S. frugiperda* cells 24 h prior to infection with the Bac-eGFP bacmid recombinant. Quantitative analysis of eGFP protein levels by fluorometry indicated that eGFP expression was maximally reduced (75%) at 24 h post-infection. However, interference efficiency decreased in correlation with the days after infection and only 54% inhibition was obtained at 48 h post-infection. These results are not entirely unexpected, since transfection of the insect cells with siGFP-22 prevented eGFP expression (*i.e.* favouring eGFP mRNA degradation) and would thus not be expected to influence viral infection. Therefore, cells initially infected would yield progeny viruses that can continue the infectious cycle. Considering that bacmid is highly cytolytic and that untreated cells may also be infected in subsequent rounds of infection, resulting in high levels of eGFP expression, it is possible that these factors may aid in abrogating the RNAi effect over time. Hence, the level of eGFP transgene inhibition was much lower at 48 compared to 24 h post-infection. Furthermore, although siRNAs have been reported to remain stable in insect (Sf-21) cells for over a week (Valdes *et al.*, 2003), it is conceivable that degradation may take place after the double-stranded siRNA is disrupted by a helicase or other as yet unidentified mechanism. Despite not having obtained complete inhibition of eGFP transgene expression in cells transfected with siGFP-22, the results are nevertheless in agreement with those reported by Agrawal *et al*. (2004). Using dsRNAs of 0.75 and 2.8 kb, Agrawal *et al.* (2004) reported a reduction of *ca.* 70% in aminopeptidase N expression levels in *S. frugiperda* cells infected with a recombinant baculovirus. Notably, this reduction in expression was only observed when the dsRNA was added to the cell culture at 2 h post-infection, and no reduction in the expression level of the protein was obtained when the dsRNA was added at 14 h post-infection. Similarly, in this study, eGFP expression was only slightly reduced (*ca.* 26-28%) when the siGFP-22 transfected cells were infected with the Bac-eGFP bacmid recombinant at 48 h post-transfection. The results obtained in this study and those reported by Agrawal *et al.* (2004) are in contrast to those reported by Valdes *et al.* (2003), whom reported that eGFP expression was greatly reduced (91%) by transfecting the cells with dsRNA (700 bp) corresponding to the entire eGFP gene 48 h prior to recombinant baculovirus infection. The apparently discordant results amongst the respective studies may, however, be due to differences in experimental design and method of analysis.

In conclusion, the results obtained in this part of the investigation indicated that eGFP expressed in *S. frugiperda* cells by recombinant bacmid infection could be silenced successfully using a cognate siRNA. This study therefore paves the way for future application of RNAi to investigate the role of specific AHSV proteins, *e.g.* NS3 and VP5, in virus virulence or to explore the assembly of AHSV structural proteins into core- and virus-like particles by making use of siRNAs that silence

expression of the structural proteins in *S. frugiperda* cells that have been co-infected with recombinant bacmids expressing the respective structural proteins.

CHAPTER THREE

INHIBITION OF AFRICAN HORSESICKNESS VIRUS VP7 PROTEIN EXPRESSION BY SMALL INTERFERING RNA IN MAMMALIAN CELLS

3.1 INTRODUCTION

For more than a decade, targeted inhibition of mammalian gene expression has been achieved primarily by approaches such as homologous recombination, antisense oligonucleotides and catalytic RNA molecules (ribozymes) (Scanlon *et al.*, 1995; Rosen and Gretch 1999; Nahreini *et al*., 2004; Robishaw *et al*., 2004). However, in the past two to three years, RNA interference (RNAi) has emerged as the primary means by which specific genes in mammalian systems can be suppressed or silenced. RNAi, a process by which double-stranded (ds) RNA directs sequence-specific degradation of a cognate mRNA, was first discovered in the nematode *Caenorhabditis elegans* (Fire *et al*., 1998; Montgomery *et al*., 1998). It has since become clear that RNAi phenomena are present in evolutionary diverse organisms including plants, fungi and metazoans (Agrawal *et al*., 2003; Meister and Tuschl, 2004). Subsequent biochemical and genetic analysis have provided a mechanistic understanding of RNAi-mediated gene silencing. In the first step, long dsRNA is recognized by a nuclease in the RNase III family, known as Dicer, which cleaves the dsRNA into small interfering RNAs (siRNAs) of 21-23 nt. These siRNAs are incorporated into a multi-component nuclease complex, known as RISC, which is then responsible for the destruction of the cognate mRNAs (Meister and Tuschl, 2004).

Despite its utility in diverse 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 (Manche *et al*., 1992; Williams, 1997; Stark *et al.*, 1998). Predominant amongst the responses triggered is activation of dsRNA-activated protein kinase (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 (Minks *et al*., 1979). This problem, however, was resolved when it was shown that in mammalian cells, RNAi can be triggered by introducing synthetic 21- to 23-nt siRNA duplexes, rather than a long dsRNA, into the cells (Elbashir *et al*., 2001b; Caplen *et al.*, 2001). The 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, which catalyzes the initiation step of RNAi. This opened the door to RNAi approaches in mammalian cells, albeit that the silencing effect is transient.

Although synthetic siRNAs can induce efficient and sequence-specific gene silencing after transfection into mammalian cells, this approach is limited by the transient nature of the gene

silencing. Consequently, several plasmid and viral vectors have been developed that allow for endogenous expression of small hairpin (sh) RNAs bearing a fold-back stem-loop structure, which can be converted by Dicer into functional siRNAs. Constitutive expression of shRNAs by RNA polymerase III U6 and H1 promoters has been reported to result in efficient and prolonged suppression of targeted genes (Brummelkamp *et al*., 2002; Yu *et al.*, 2002; Rubinson *et al*., 2003; Gupta *et al*., 2004). However, reports have indicated that shRNAs may be less effective in mediating gene silencing than synthetic siRNAs (Paddison *et al.*, 2002) and, in some cases, they have been reported to activate an interferon response similar to that described with long dsRNA (Bridge *et al*., 2003; Kim *et al.*, 2004).

At present, it is not possible to predict with complete certainty the degree of gene suppression a particular siRNA will produce. However, a number of recommendations have been made, which may increase the probability of producing an effective siRNA (Elbashir *et al*., 2001a; Elbashir *et al*., 2001b; Elbashir *et al*., 2001c; Reynolds *et al*., 2004). The two most important factors influencing siRNA efficacy appear to be the target site within the gene and the structural characteristics of the siRNA. The target site should be located at least 100-200 nt from the AUG initiation codon and within 50-100 nt from the termination codon to avoid interference by RNA-regulatory proteins that bind to the 5' or 3' untranslated regions (UTRs). Regarding the structural characteristics of the siRNA, it has been reported that siRNA duplexes of 21 nt with 3'-d(TT) or 3'-d(UU) overhangs are the most effective, as they resemble the cleavage products produced by Dicer during the initiation step of RNAi and serves to safeguard the siRNAs from exonuclease activity (Elbashir *et al*., 2001b; 2001c; Mittal, 2004). Studies on the thermodynamic characteristics of siRNAs have revealed three criteria important for siRNA functionality, *i.e.* moderate to low G+C-content (45-55%) to facilitate interaction with the RISC nuclease complex and unwinding; low internal stability at the 5' antisense strand to promote antisense strand-selection by RISC, which needs to be incorporated into RISC to direct cleavage of the target mRNA (Khvorova *et al*., 2003; Schwarz *et al*., 2003); and absence of internal repeats or palindromic sequences to increase the concentration of functional, stable hairpins (Reynolds *et al*., 2004).

There has been an increase in the number of reports demonstrating that siRNAs can, amongst other, hinder the assembly of viral particles and that they are useful in elucidating the role of viral and cellular genes in host-virus interactions (reviewed in Tan and Yan, 2004). In one such a study, Déctor *et al.* (2002) used siRNA to efficiently inhibit synthesis of the rotavirus VP4 protein, which functions in the attachment of virus particles to cell receptors and in the penetration of the virions into the cell. The results indicated that the yield of viral progeny was reduced and most of the virus

particles purified from these cells were poorly infectious. The virion of African horsesickness virus (AHSV), an orbivirus that like rotavirus is a member of the *Reoviridae* family, is composed of seven structural proteins organized into a double-layered capsid containing the ten dsRNA segments of the AHSV genome (Bremer *et al.*, 1990). The icosahedral core is composed of two major structural proteins, VP3 and VP7 (Huismans *et al.*, 1987; Prasad *et al.*, 1992; Stuart *et al.*, 1998). For bluetongue virus (BTV), the prototype orbivirus, it has been reported that the core particles are highly infectious for the *Culicoides* insect vector and *Culicoides*-derived cell culture (Mertens *et al.*, 1996). Since VP7 is the outermost core protein, and thus the most accessible protein of the BTV core, these results suggest that it may participate in vector cell entry. Consequently, targeting the AHSV VP7 protein for siRNA-mediated silencing may not only provide insights into core and virus particle assembly, but may also provide insights into vector-virus interactions.

In the previous Chapter, attempts were made to develop and establish an RNAi assay whereby transgene expression in the bacmid/insect cell expression system could be silenced. Since complete inhibition of eGFP transgene expression could not be obtained and based on the above, indicating that RNAi in mammalian cell cultures is tractable, it was next investigated whether gene-specific silencing in mammalian cells can be achieved. Consequently, the primary aim of this part of the investigation was to develop an RNAi assay whereby expression of the VP7 gene of AHSV-9 could be silenced *in vitro* in BHK-21 mammalian cells. For this purpose a recombinant mammalian expression vector expressing a chimeric AHSV VP7-eGFP protein was constructed. In addition, different siRNAs targeting the AHSV VP7 mRNA were designed and their efficacy to inhibit VP7-eGFP gene expression was compared.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial strains and plasmids

The *E. coli* strains were routinely cultured in LB broth (1% [w/v] tryptone; 1% [w/v] NaCl; 0.5% [w/v] yeast extract; pH 7.4) (Sambrook *et al.*, 1989) at 37˚C with shaking at 200 rpm, and maintained at 4˚C on LB agar (LB broth containing 1.2% [w/v] bacteriological agar) or at -70˚C as glycerol cultures. For plasmid DNA selection and maintenance in *E. coli*, the culture medium was supplemented with 100 µg/ml of ampicillin (Roche Diagnostics). Recombinant plasmids pGEM-VP7 and pGEM-eGFP, containing a full-length copy of the AHSV-9 VP7 gene and enhanced green fluorescent protein (eGFP) gene, respectively, were constructed and characterized previously (Chapter 2). A recombinant pCMV-Script® construct containing the eGFP gene was obtained from Ms J. Roos, Department of Microbiology and Plant Pathology, University of

Pretoria. The pGEM®-T Easy cloning vector system was obtained from Promega and the pCMV-Script® mammalian expression vector was obtained from Stratagene.

3.2.2 Construction of a recombinant pCMV-Script® mammalian expression vector

All molecular cloning techniques employed in the construction of a recombinant pCMV-Script® mammalian expression vector, harbouring a chimeric AHSV-9 VP7-eGFP gene, was performed according to the procedures described in Chapter 2 (Sections 2.2.2 through 2.2.8). All plasmid constructs were confirmed by restriction endonuclease digestions using agarose gel electrophoresis and by nucleotide sequencing.

- **pCMV-VP7-eGFP**

Towards construction of the recombinant mammalian expression vector pCMV-VP7-eGFP, a truncated VP7 gene lacking a stop codon was PCR-amplified, as described previously (Section 2.2.3.2). For this purpose, plasmid pGEM-VP7 was used as template DNA in the PCR together with oligonucleotides VP7-F (5'-CAC**agatct**ATGGACGCGATAGC-3') and VP7-RHind (5'-CAC**aagctt**GTGGTAGGCTGCTA-3'), which contain a *Bgl*II and *Hind*III site, respectively (bold). The amplicon was cloned into pGEM®-T Easy vector DNA to generate recombinant plasmid pGEM-VP7trunc. The insert DNA was subsequently recovered by digestion with both *Bgl*II and *Hind*III, and cloned into the *Bam*HI and *Hind*III sites of pCMV-Script® to yield pCMV-VP7trunc. Oligonucleotides eGFP-FHind (5'-CAC**aagctt**ATGGTGAGCAAGG-3') and eGFP-RXho (5'-CAC**ctcgag**TTACTTGTACAGCTCGT-3'), which contain a *Hind*III and *Xho*I site, respectively (bold), were subsequently used with plasmid pGEM-eGFP as template DNA to PCR-amplify a 720-bp amplicon, which was cloned into pGEM®-T Easy vector DNA to generate pGEM-eGFPchim. To complete construction of pCMV-VP7-eGFP, the cloned DNA fragment was recovered by digestion with both *Hind*III and *Xho*I and cloned into identically digested pCMV-VP7trunc. The cloning strategy employed in the construction of pCMV-VP7-eGFP is indicated in Fig. 3.1.

3.2.3 Plasmid isolation and purification

3.2.3.1 Plasmid DNA extraction

Large-scale plasmid extractions were performed according to the procedures described by Sambrook *et al.* (1989) with the following modifications. Five ml of an overnight culture was inoculated into 500 ml LB broth, containing the appropriate antibiotic, and incubated at 37˚C for 20 h with active aeration (200 rpm). The bacterial cells were collected by centrifugation at 5 000 rpm for 15 min in a HS-4 rotor, using a Sorvall® RCSB Plus centrifuge (DuPont), and rinsed once with 76 ml of 1 × STE buffer (0.1 M NaCl; 10 mM Tris-HCl; 1 mM EDTA; pH 8.0). The cell pellet was suspended in 40 ml of Solution I (50 mM glucose; 10 mM EDTA; 25 mM Tris; pH 8.0) and incubated on ice for 30 min. The cells were lysed by the addition of 80 ml of Solution II (0.2 N NaOH; 1% [w/v] SDS) and after incubation on ice for 5 min, 60 ml of Solution III (3 M NaOAc; pH 4.8) was added. After incubation on ice for 1 h, the insoluble aggregate that formed was removed by centrifugation at 10 000 rpm for 15 min. The plasmid-containing supernatant was recovered and

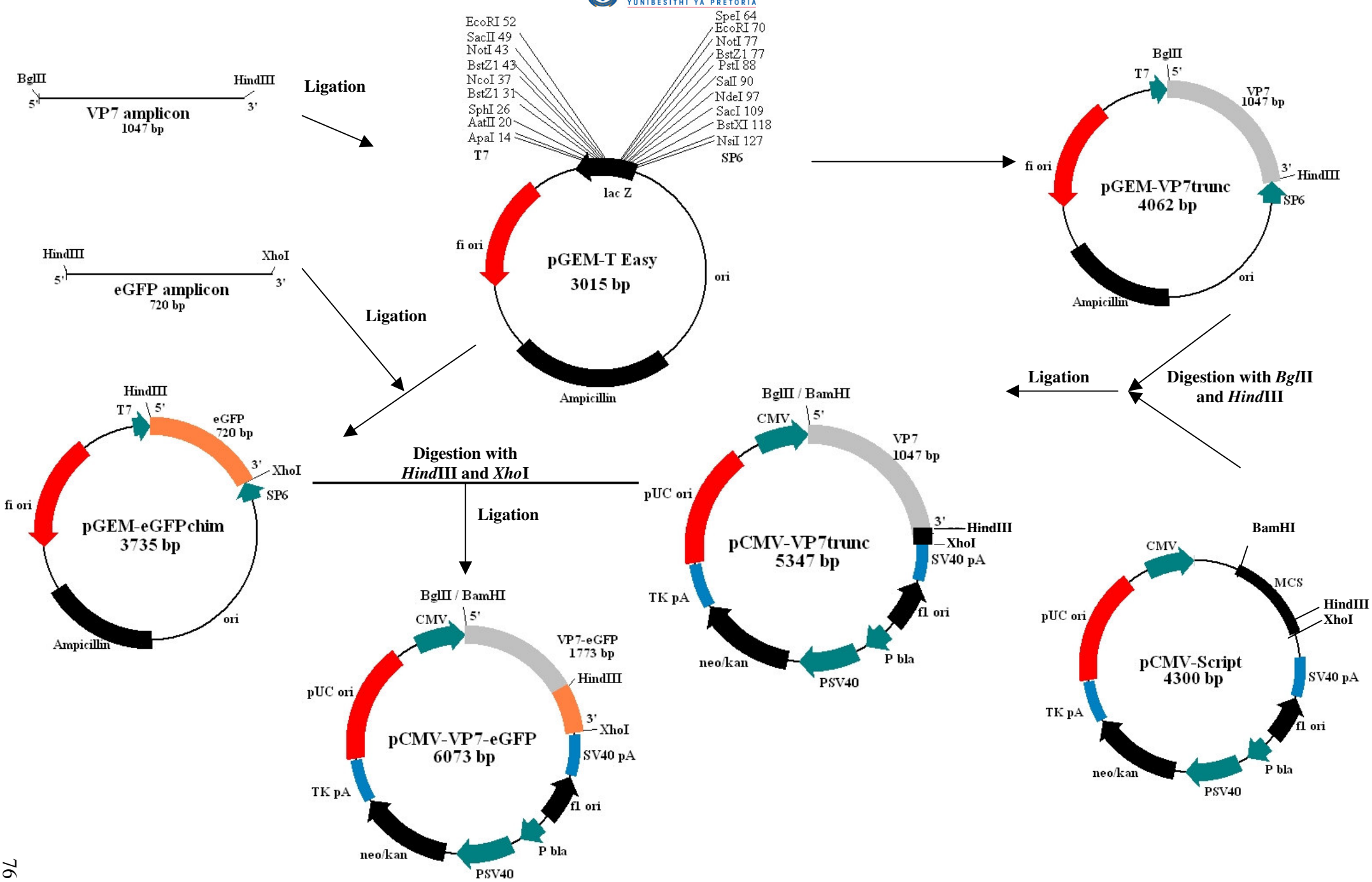

Fig. 3.1 **Construction of the AHSV-9 VP7-eGFP chimeric gene in pCMV-Script®.**

filtered through Whatman® filter paper, after which the plasmid DNA was precipitated by the addition of 2.5 volumes 96% ethanol and incubation overnight at -20˚C. The precipitated plasmid DNA was collected by centrifugation at 10 000 rpm for 15 min, suspended in 60 ml UHQ water and 25 ml of ice-cold 7.5 M NH_4OAc was added. After incubation on ice for at least 30 min, the high-molecular-weight RNA precipitate was removed by centrifugation at 10 000 rpm for 10 min. The plasmid DNA was subsequently recovered from the supernatant by ethanol precipitation, as described above. The DNA pellet was rinsed with 70% ethanol, vacuum-dried and suspended in 300 µl of 1 × TE buffer (10 mM Tris-HCl; 1 mM EDTA; pH 8.0).

3.2.3.2 Purification of plasmid DNA

Plasmid DNA was purified by centrifugation in CsCl-ethidium bromide density gradients, as described by Sambrook *et al.* (1989) with the following modifications. The volume of the large-scale plasmid DNA suspension was adjusted to 1 ml with 1 × TE. An amount of 3.234 g of CsCl was added to 2 ml of 1 × TE and the solution was incubated at 30˚C to facilitate the dissolution of the salt. The plasmid DNA and CsCl solutions were mixed in a 5-ml polyallomer centrifuge tube (Beckman) and 300 µl of a 10 mg/ml ethidium bromide solution was then added. The gradient was centrifuged at 38 000 rpm for 40 h at 20˚C in a Beckman SW 55Ti rotor, using a Sorvall® Ultra Pro80 centrifuge (DuPont). The tubes were viewed under UV light and the plasmid-containing lower band was removed by inserting a hypodermic needle (18 × 1.5 gauge), connected to a syringe, into the tube just below the lower band. The plasmid DNA solution was transferred to a 30-ml Corex tube and diluted with 4 volumes of 1 × TE. The ethidium bromide was removed from the DNA-containing solution by the addition of an equal volume water-saturated *n*-butanol. The phases were mixed by vortexing and separated by centrifugation at 1 500 rpm for 3 min at room temperature. The extraction was repeated until all traces of ethidium bromide were removed. The lower aqueous phase was then transferred to a 15-ml Greiner tube and the volume was adjusted to 4 ml with 1 × TE. Plasmid DNA, free of protein and RNA contaminants, was precipitated by the addition of 2 volumes 96% ethanol. Following incubation at 4˚C for 1 h, the precipitated plasmid DNA was recovered by centrifugation, rinsed with 70% ethanol and dried under vacuum prior to being suspended in 300 µl UHQ water.

3.2.3.3 Quantification of plasmid DNA

An aliquot (1 µl) of purified plasmid DNA, together with DNA of known concentration, was analyzed by electrophoresis on an 0.8% agarose gel, and the plasmid DNA concentration was determined using the VersaDoc™ Imaging System and Quantity One® 1-D Analysis software program (BioRad).

3.2.4 Transient expression of VP7-eGFP and eGFP in BHK-21 cells

3.2.4.1 Cells and culture conditions

Baby hamster kidney-21 (BHK-21) cells were propagated and maintained as monolayers in 75-cm^2 tissue culture flasks, and cultured in Minimum Essential Medium (MEM) Eagles base (Highveld Biological) supplemented with 2.5% or 5% (v/v) foetal bovine serum (FBS) and antibiotics (1 × penicillin, streptomycin, fungizone). The flasks were incubated at 37˚C in a humidified incubator with a constant supply of 5% CO_2.

3.2.4.2 Transfection of BHK-21 cells

BHK-21 cells were transfected with purified recombinant pCMV-Script® plasmid DNA using the Lipofectamine™ 2000 reagent (Invitrogen) according to the manufacturer's instructions. The cells were seeded in 35-mm-diameter wells to reach 80% confluency within 24 h of incubation at 37˚C in the presence of 5% CO_2. For each transfection, 4 µg of purified plasmid DNA and 10 µl of the Lipofectamine™ 2000 reagent was separately diluted in 250 µl of antibiotic- and serum-free MEM medium. Following incubation at room temperature for 5 min, the two solutions were mixed and then incubated at room temperature for 20 min to allow the formation of DNA-lipofectamine complexes. The cell monolayers were subsequently prepared for transfection by rinsing the cells three times with 2 ml of antibiotic-free MEM medium containing 2.5% (v/v) FBS. After addition of 1.5 ml of the same medium, the cells were overlayed with the DNA-lipofectamine complexes and the tissue culture dishes were incubated for 48 h at 37˚C in a CO_2 incubator. Mock-transfected cells and cells transfected with parental pCMV-Script® plasmid DNA were included as controls. Following incubation, transgene expression was assayed by epifluorescence microscopy as described below.

3.2.5 RNA interference assays in BHK-21 cells

3.2.5.1 Small interfering RNAs (siRNAs)

In addition to siGFP-22, a siRNA directed against eGFP mRNA, and scrambled (negative control) siRNA, which was designed by scrambling the sequence of siGFP-22 (Chapter 2), additional siRNAs directed specifically against the AHSV-9 VP7 mRNA was designed. Target sites chosen for these siRNAs were identified using Ambion siRNA Target Finder (available at http://www.ambion.com), Qiagen siRNA Design Tool (available at http://www.qiagen.com) and Oligoengine (available at http://www.oligoengine.com). The target sites were subsequently compared to the entries of the GenBank database by making use of the BLASTN program (Altschul *et al.*, 1997) available on the National Centre for Biotechnology Information web page (http://www.ncbi.nlm.nih.gov/). The accessibility of the remaining target sites was evaluated by RNA secondary structure analysis of the VP7 and chimeric VP7-eGFP mRNA, using MFOLD hybridization and folding software v.3.1 (Zuker, 2003). Based on the results obtained from these *in silico* analyses (Fig. 3.2), the design of the siRNAs was refined, following the design recommendations of Elbashir *et al*. (2001b).

Table 3.1 Gene target sites, and sense and antisense sequence of siRNAs directed against AHSV-9 VP7 mRNA

siRNA	Coding region targeted	DNA target sequence	siRNA sequence *	G+C-content	Gene
siVP7-336 (Sense)	336-356	5' - AACGGGTCAGATGCAAACATT - 3'	5' - CGGGUCAGAUGCAAACAUUdTT - 3'	42.9%	AHSV-9 VP7
siVP7-336 (Antisense)	336-356	5' - AACGGGTCAGATGCAAACATT - 3'	5' - AAUGUUUGCAUCUGACCCGdTT - 3'	42.9%	AHSV-9 VP7
siVP7-441 (Sense)	441-461	5' - AACGCGTGGTGGGTACATCAA - 3'	5' - CGCGUGGUGGGUACAUCAAdTT - 3'	52.4%	AHSV-9 VP7
siVP7-441 (Antisense)	441-461	5' - AACGCGTGGTGGGTACATCAA - 3'	5' - UUGAUGUACCCACCACGCGdTT - 3'	52.4%	AHSV-9 VP7

* The AHSV-9 VP7 open reading frame was selected as the targeted region, avoiding regions within 100 nt from the initiation and termination codons. The siRNA was 21 nt in length, with the sequence motif 5'-(N_{19})TT-3', consisting of a 19-nt duplex RNA and 2-nt deoxythymidine 3'-end overhangs. Target regions with a G+C-content between 30 to 60%, without stretches of four or more bases or tandem repeats, were selected.

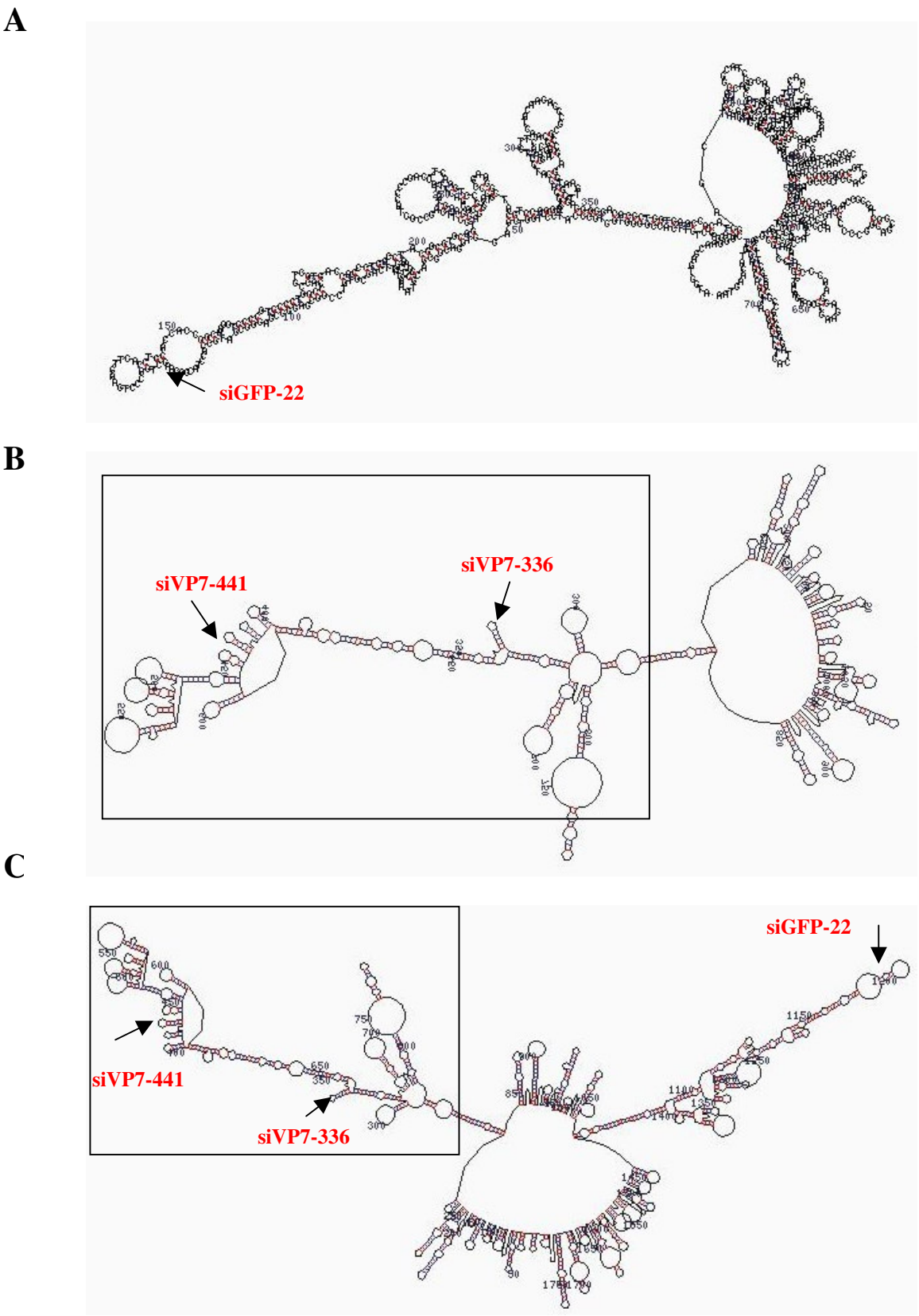

Fig. 3.2 **Local secondary structure of the enhanced green fluorescent protein (eGFP) (A), AHSV-9 VP7 (B) and VP7-eGFP (C) mRNAs, as predicted by the MFOLD software program (Zuker, 2003). The conserved regions between VP7 and VP7-eGFP mRNA are blocked, with arrows indicating the different regions targeted for inhibition by siRNAs.**

The siRNAs used in this part of the study are shown in Table 3.1 and were obtained from Qiagen. Stock solutions (20 µM) of the respective siRNAs were prepared by suspension of the lyophilized complexes in RNase-free buffer (100 mM potassium acetate; 30 mM HEPES-KOH; 2 mM magnesium acetate; pH 7.4). To disrupt higher aggregates that may have formed during lyophilization, the tubes were heated to 90˚C for 1 min and then incubated at 37˚C for 1 h. The siRNAs were stored at -20˚C until required.

3.2.5.2 Cotransfection of BHK-21 cells with siRNA and recombinant pCMV-Script® mammalian expression vectors

BHK-21 cells were seeded in 24-well tissue culture dishes to reach 50% confluency within 24 h of incubation at 37˚C in the presence of 5% CO_2. The recombinant pCMV-Script® plasmids containing either the eGFP gene (pCMV-eGFP) or chimeric VP7-eGFP gene (pCMV-VP7-eGFP) were subsequently cotransfected together with the siRNA into BHK-21 cells using Lipofectamine™ 2000 reagent (Invitrogen). Briefly, 0.8 µg of CsCl-purified plasmid DNA and 2 µl of Lipofectamine™ 2000 reagent were each diluted in 50 µl of serum-and antibiotic-free MEM medium, incubated at room temperature for 5 min and then mixed to allow the formation of DNA-lipofectamine complexes. Seperately, 20 pmol of the siRNA and 1 µl of Lipofectamine™ 2000 reagent were each diluted in 50 µl MEM medium (without serum and antibiotics) and, following incubation at room temperature for 5 min, were mixed to allow formation of RNA-lipofectamine complexes. After incubation at room temperature for 20 min, the two solutions were mixed and then used to overlay the BHK-21 cell monolayers, which had been prepared by rinsing the cells three times with 500 µl of antibiotic-free MEM medium containing 2.5% (v/v) FBS. After the addition of 300 µl of the same medium, the tissue culture dishes were incubated for 24 h at 37˚C in a CO_2 incubator. Mock-transfected BHK-21 cells and cells transfected with the parental pCMV-Script® vector were included as controls.

3.2.5.3 Analysis and quantification of eGFP and VP7-eGFP expression in BHK-21 cells

The BHK-21 cell monolayers were observed at 24 and 48 h post-transfection for the expression of eGFP and VP7-eGFP on a Zeiss Axiovert 200 fluorescent microscope fitted with the no. 10 Zeiss filter set (excitation at 450-490 nm; emission at 515-565 nm). The images were captured for at least three separate microscope fields, using a Nikon DXM1200 digital camera, and analyzed with Nikon ACT-1 v.2.20 software. For fluorometry analysis, the BHK-21 cell monolayers were rinsed once with 1 × 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 trypsinized by the addition of 80 µl Trypsin Versene. Following incubation at 37˚C for 5 min, the cells were collected by centrifugation at 5 000 rpm for 10 min, rinsed with 1 × PBS and suspended in 1 ml of 1 × PBS. The relative fluorescence was determined using a Versafluor™ fluorometer (emission at 515-525 nm and excitation at 485-495 nm; BioRad).

3.3 RESULTS

3.3.1 Construction of a recombinant pCMV-Script® mammalian expression vector containing a chimeric AHSV-9 VP7-eGFP gene

Towards developing an RNAi strategy whereby expression of VP7 of AHSV-9 could be silenced in mammalian cells, a chimeric VP7-eGFP gene was constructed. The eGFP reporter gene was shown previously in this study (Chapter 2) to provide a simple and impartial model for assessing the efficacy of gene silencing by analysis at the protein level visually and quantitatively by fluorometry. In addition, the availability of a fluorescently tagged VP7 protein, coupled with the above assay methods, would greatly facilitate the rapid screening of different VP7-specific siRNAs for their ability to silence gene expression. The chimeric gene was constructed by making use of a strategy whereby the eGFP gene was fused in-frame to the C-terminus of the VP7 gene. For this purpose, the VP7 gene was PCR-amplified using a 3'-specific oligonucleotide designed to amplify a truncated AHSV-9 VP7 gene, which lacked the TAG stop codon. Furthermore, this oligonucleotide and the 5'-specific oligonucleotide used to PCR-amplify the eGFP gene were designed to contain a unique *Hind*III restriction endonuclease recognition site at their 3'- and 5'-ends, respectively, which following restriction endonuclease digestion of the respective DNA fragments, could be ligated to yield the desired VP7-eGFP chimeric gene (Fig. 3.1).

Towards construction of the VP7-eGFP gene both the VP7 and eGFP genes were PCR-amplified, using recombinant pGEM®-T Easy constructs as template DNA and the appropriate oligonucleotides (Section 3.2.2), to generate amplicons of *ca.* 1.047 kb and 720 bp, respectively. The amplicons were cloned into pGEM®-T Easy vector DNA and then characterized by nucleotide sequencing to confirm their integrity. Having confirmed that the stop codon was deleted, the truncated VP7 gene was recovered and cloned into the *Bam*HI and *Hind*III sites of pCMV-Script®, thereby generating pCMV-VP7trunc. To complete construction of pCMV-VP7-eGFP (Fig. 3.3A), the eGFP gene was recovered from the recombinant pGEM®-T Easy plasmid by digestion with both *Hind*III and *Xho*I and cloned into identically prepared pCMV-VP7trunc. The recombinant plasmid DNA was characterized by agarose gel electrophoresis following restriction endonuclease digestion.

To verify the presence of the VP7-specific insert DNA, plasmid pCMV-VP7trunc was digested with *Bam*HI and *Hind*III and yielded expected bands corresponding to *ca.* 4.375 kb and 972 bp (Fig. 3.3B, lane 6). The recombinant plasmid pCMV-VP7-eGFP was subsequently characterized by digestion with *Xho*I and *Hind*III or *Bam*HI. Whereas digestion of the recombinant plasmid with

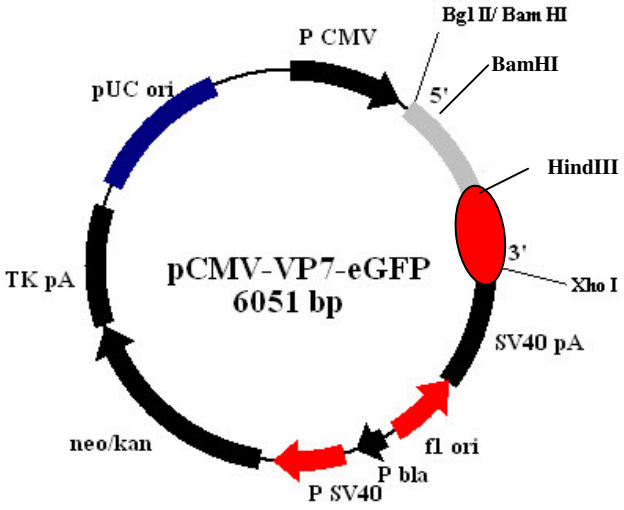

Fig. 3.3A **Plasmid map of the recombinant plasmid pCMV-VP7-eGFP harbouring a chimeric VP7-eGFP gene. The VP7 gene of AHSV-9 is indicated in grey and the eGFP gene as a red circle.**

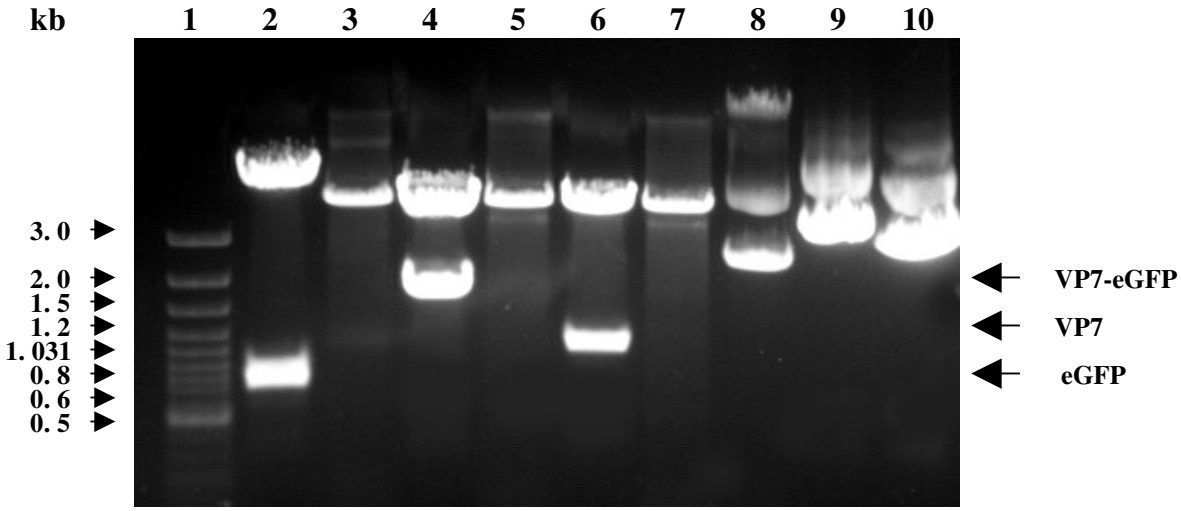

Fig. 3.3B **Agarose gel electrophoretic analysis of recombinant plasmid pCMV-VP7-eGFP and the intermediate plasmid pCMV-VP7trunc used in its construction. Lane 1, DNA molecular weight marker; lane 2, recombinant plasmid pCMV-VP7-eGFP vector DNA digested with *Xho*I and *Hind*III; lane 3, parental pCMV-Script® vector DNA digested with *Xho*I and *Hind*III; lane 4, recombinant plasmid pCMV-VP7-eGFP vector DNA digested with *Xho*I and *Bam*HI; lane 5, parental pCMV-Script® vector DNA digested with *Xho*I and *Bam*HI; lane 6, recombinant plasmid pCMV-VP7trunc vector DNA digested with *Bam*HI and *Hind*III; lane 7, parental pCMV-Script® vector DNA digested with *Bam*HI and *Hind*III; lane 8, uncut parental pCMV-Script® vector DNA; lane 9, uncut parental pCMV-VP7-eGFP vector DNA; lane 10, uncut pCMV-VP7trunc vector DNA. The sizes of the DNA molecular weight marker, GeneRuler™ 100-bp DNA Ladder Plus (Fermentas), are indicated to the left of the figure.**

*Xho*I and *Hind*III yielded two DNA fragments corresponding to the size of the pCMV-VP7trunc vector DNA (5.347 kb) and the cloned eGFP gene (720 bp) (Fig. 3.3B, lane 2), digestion with *Xho*I and *Bam*HI resulted in the excision of a 1.698-kb DNA fragment corresponding in size to the chimeric VP7-eGFP gene (Fig. 3.3B, lane 4). These results therefore confirmed that a VP7-eGFP chimeric gene had been constructed successfully in the mammalian expression vector pCMV-Script®.

Since the *Taq* DNA polymerase used to PCR-amplify the VP7 and eGFP genes lacked a proofreading activity, the cloned insert DNA was finally characterized by nucleotide sequencing since it has been reported that a single mismatch between the siRNA and target mRNA can abolish siRNA-mediated gene silencing (Elbashir *et al.*, 2001a). Except for deletion of the stop codon from VP7 and the introduction of six nucleotides specifying the newly introduced *Hind*III site (to allow in-frame fusion of the VP7 and eGFP genes), no other differences were observed between the sequence of the chimeric VP7-eGFP gene and those of the full-length VP7 and eGFP genes determined previously (Chapter 2). In addition, analysis of the deduced amino acid sequence confirmed that the open reading frame of the chimeric VP7-eGFP protein was maintained.

3.3.2 Transient expression of eGFP and VP7-eGFP in BHK-21 cells

Prior to transfection, the parental pCMV-Script® and recombinant mammalian expression vectors pCMV-VP7-eGFP and pCMV-eGFP (obtained from Ms J. Roos, Department of Microbiology and Plant Pathology, University of Pretoria) were CsCl-purified, since impurities in the plasmid preparations may influence transfection efficiencies. BHK-21 cell monolayers were subsequently transfected with the respective constructs, using the Lipofectamine™ 2000 reagent, and examined at 48 h post-transfection by epifluorescence microscopy.

Examination of the cell monolayers by epifluorescence microscopy (Fig. 3.4) indicated that BHK-21 cells transfected with pCMV-eGFP and pCMV-VP7-eGFP fluoresced brightly, whilst no fluorescence was observed in mock-transfected cells or in cells transfected with the parental pCMV-Script® vector. To confirm expression of the VP7-eGFP and eGFP proteins, cell lysates were prepared and analyzed by SDS-PAGE and immunoblot analyses. No uniquely expressed proteins were observed in the cells transfected with the recombinant pCMV-Script® expression vectors compared to mock-transfected cells, whilst immunoblot analysis performed with a polyclonal anti-eGFP antibody and an AHSV-9 antiserum yielded several non-specific immuno-reactive bands (results not shown). The lack in detecting the expressed proteins by SDS-PAGE and immunoblot analyses is most likely due to the low levels of transient gene expression.

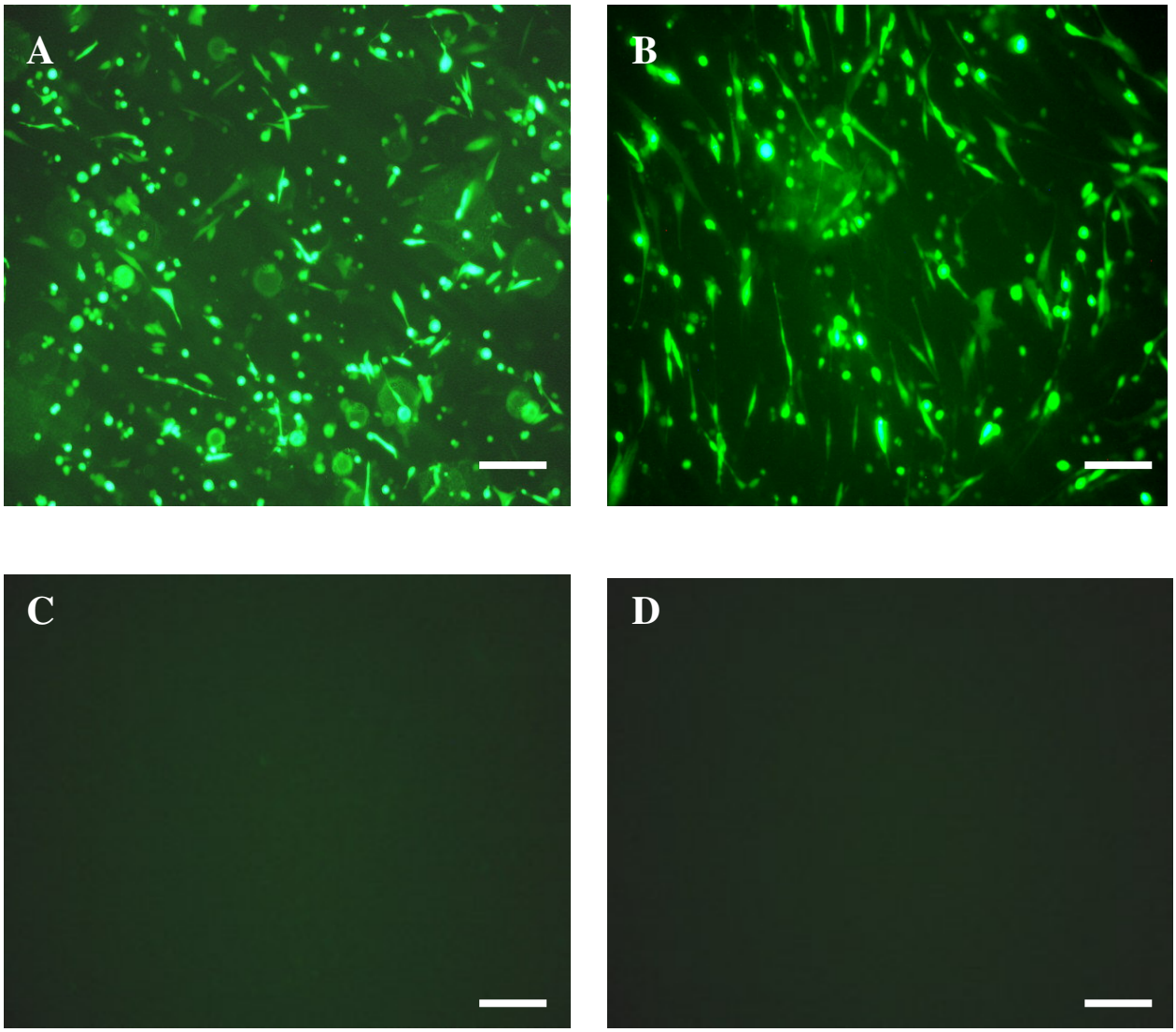

Fig. 3.4 **Epifluorescence microscopy of eGFP and VP7-eGFP gene expression in BHK-21 cells. BHK-21 cells transfected with 4 µg pCMV-eGFP (A) and pCMV-VP7-eGFP (B) were analyzed for protein expression 48 h post-transfection, using a Zeiss Axiovert fluorescent microscope. Mock-transfected (C) and pCMV-Script ®-transfected (D) BHK-21 cells were included as controls. Bar = 100 µm.**

Despite the above results, note should, however, be taken that the sources of the eGFP and VP7-eGFP genes used in the construction of the recombinant mammalian expression vectors were identical to those used in the construction of recombinant bacmids (Chapter 2). Using the respective bacmid recombinants, it was shown that these genes were successfully expressed in *S. frugiperda* cells and that the synthesized proteins reacted with either an AHSV-9 antiserum or anti-eGFP antibody (Fig. 2.8). Therefore, the fluorescence observed in BHK-21 cells transfected with either pCMV-eGFP or pCMV-VP7-eGFP provides strong evidence for the successful expression of the eGFP and VP7-eGFP proteins in BHK-21 cells.

3.3.3 Inhibition of eGFP and VP7-eGFP expression by an eGFP gene-specific siRNA in BHK-21 cells

To determine whether siRNAs could be used to inhibit AHSV-9 VP7 gene expression in BHK-21 cells, RNAi in these cells was first assessed by using eGFP as reporter gene and siRNA directed against the eGFP mRNA. As a control, scrambled siRNA, non-homologous to the targeted gene, was used to evaluate non-specific effects on eGFP gene expression and to exclude any changes in gene expression that may have resulted from the siRNA delivery method. In the RNAi assay, the pCMV-eGFP and pCMV-VP7-eGFP plasmids were cotransfected into BHK-21 cells with either the homologous or heterologous siRNA, using the optimized assay conditions, as described under Materials and Methods (Section 3.2.5). The BHK-21 cell monolayers were analyzed at 24 h post-transfection by fluorescence microscopy and the eGFP fluorescence was quantified by fluorometry. The results of these analyses are shown in Fig. 3.5 and Fig. 3.6, respectively.

Examination of the BHK-21 cell monolayers by fluorescence microscopy indicated extensive fluorescence of cells transfected with the pCMV-eGFP or pCMV-VP7-eGFP plasmids, whereas cotransfection of these plasmids with siGFP-22, in contrast, greatly abrogated fluorescence. Notably, eGFP and VP7-eGFP expression in cells cotransfected with the scrambled (negative control) siRNA was comparable to that observed in cells transfected with the vector controls, suggesting that the observed RNAi effects are gene-specific (Fig. 3.5). Quantitative analyses of eGFP expression by fluorometry (Fig. 3.6) indicated that siGFP-22 inhibited eGFP expression by *ca.* 95%, whilst the scrambled (negative control) siRNA suppressed eGFP expression by only *ca.* 7%. Expression of VP7-eGFP was inhibited by *ca.* 97% in cells cotransfected with siGFP-22, whereas the scrambled (negative control) siRNA suppressed VP7-eGFP expression by *ca.* 25%. The results obtained in this section indicated that VP7-eGFP and eGFP expression could be efficiently inhibited by a gene-specific siRNA in mammalian (BHK-21) cells.

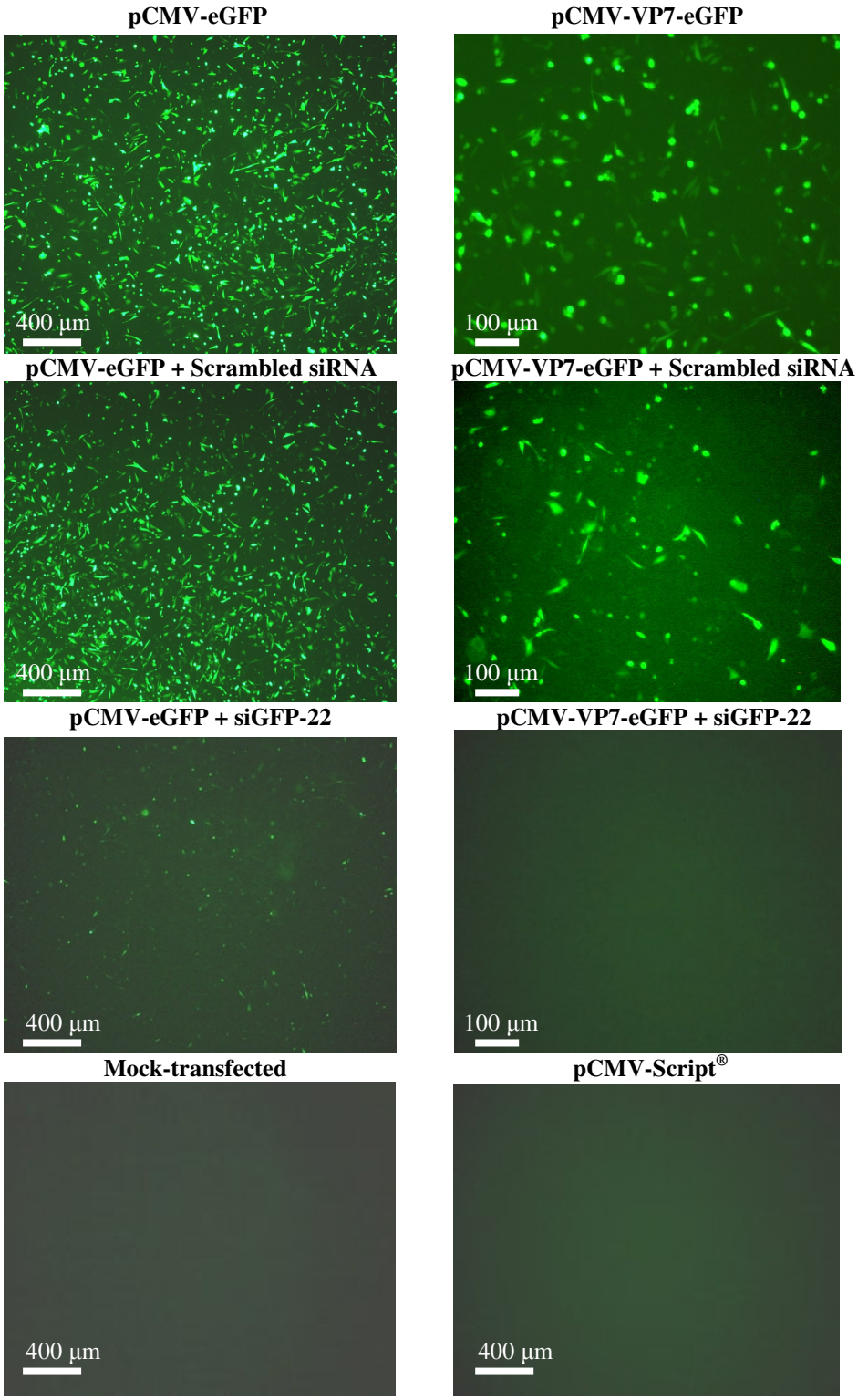

Fig. 3.5 **Silencing of eGFP and VP7-eGFP protein expression in BHK-21 cells. BHK-21 cells were transfected with either pCMV-eGFP or pCMV-VP7-eGFP plasmid DNA and cotransfected with the eGFP-specific siRNA (siGFP-22), as well as scrambled (negative control) siRNA. Mock-transfected BHK-21 cells and cells transfected with parental pCMV-Script® vector DNA are shown for comparative purposes. At 24 h post-transfection, representative fields were photographed.**

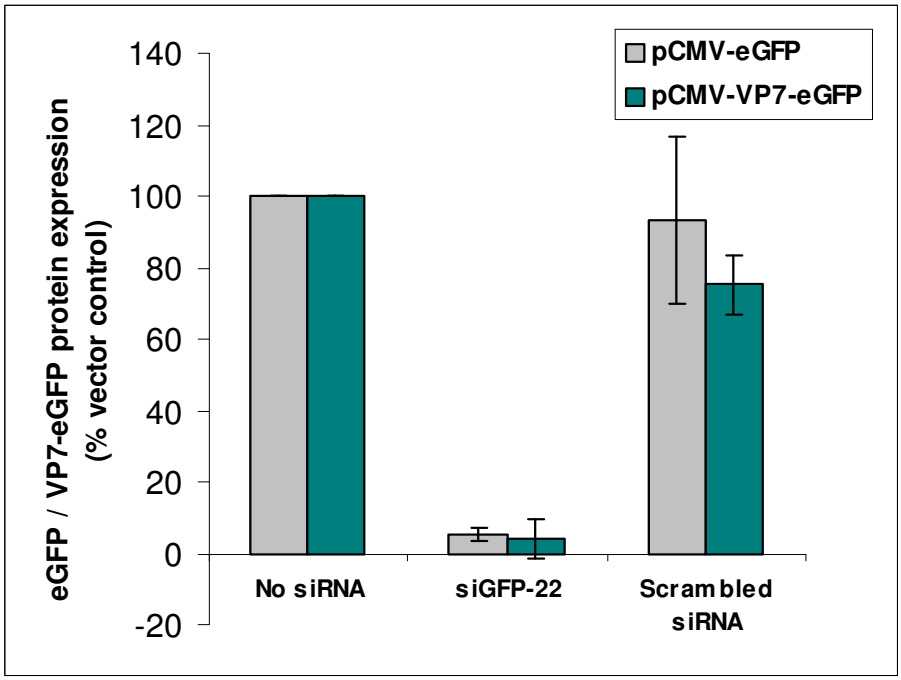

Fig. 3.6 **Silencing of eGFP and VP7-eGFP protein expression by siGFP-22 in BHK-21 cells. BHK-21 cells were transfected with either pCMV-eGFP or pCMV-VP7-eGFP plasmid DNA and cotransfected with the siGFP-22 and scrambled (negative control) siRNA. At 24 h post-transfection, cells were analyzed for eGFP expression by fluorometry and expressed as percentages of the value for cells transfected with the recombinant expression vector constructs (no siRNA controls). The data are means ± standard deviations (SD) of values from three independent experiments.**

3.3.4 Inhibition of VP7-eGFP expression by VP7 gene-specific siRNAs in BHK-21 cells

In the previous section it was shown that RNAi can be used to efficiently silence heterologous gene expression in BHK-21 cells. These studies were subsequently extended by investigating whether VP7 gene-specific siRNAs may similarly cause inhibition of AHSV-9 VP7 gene expression. Using computer-aided predictions of the RNA structure, two putative accessible sites were identified. The relevance of such analysis is, however, still uncertain since the method does not take into account either tertiary structure or RNA-protein interactions. Despite these limitations, siRNAs directed to these different sites on the AHSV-9 VP7 mRNA were designed, chemically synthesized and then tested in cell culture. The siRNAs were designated siVP7-336 and siVP7-441, respectively (Table 3.1; Fig. 3.2).

BHK-21 cells were cotransfected with pCMV-VP7-eGFP and pCMV-eGFP in the absence or presence of siVP7-336, siVP7-441 or scrambled (negative control) siRNAs. The cell monolayers were analyzed for VP7-eGFP and eGFP expression by fluorescent microscopy at 24 h post-transfection. The results obtained (Fig. 3.7) indicated that expression of the VP7-eGFP protein was diminished greatly in cells transfected with siVP7-336 and siVP7-441, when compared to cells transfected with the pCMV-VP7-eGFP vector control only. Notably, siVP7-336 appeared to inhibit VP7-eGFP gene expression slightly more efficiently than siVP7-441 since, in most transfected cells, VP7-eGFP expression was reduced to near background levels. By contrast, expression of eGFP did not appear to be inhibited in cells transfected with either siVP7-336 or siVP7-441. BHK-21 cells transfected with scrambled (negative control) siRNA strongly expressed eGFP and VP7-eGFP, which was equivalent to control cells transfected with pCMV-VP7-eGFP and pCMV-eGFP.

To determine and compare the efficacy of the respective siRNAs to silence VP7-eGFP gene expression, the cells were subsequently harvested and the expression of VP7-eGFP was analyzed quantitatively by fluorometry (Fig. 3.8). The results indicated that in cells transfected with siVP7-336 or siVP7-441, expression of VP7-eGFP was reduced by 88% and 75%, respectively (Fig. 3.8). By contrast, in cells that were transfected with scrambled (negative control) siRNA, the VP7-eGFP expression was reduced by *ca.* 18%. However, in control cells cotransfected with pCMV-eGFP and the VP7-specific siRNAs, no significant inhibition of eGFP was found (6%). From these results it was concluded that VP7-eGFP expression could be inhibited specifically by siRNAs directed against the VP7 mRNA, and that siVP7-336 is more effective than siVP7-441 in silencing VP7-eGFP gene expression.

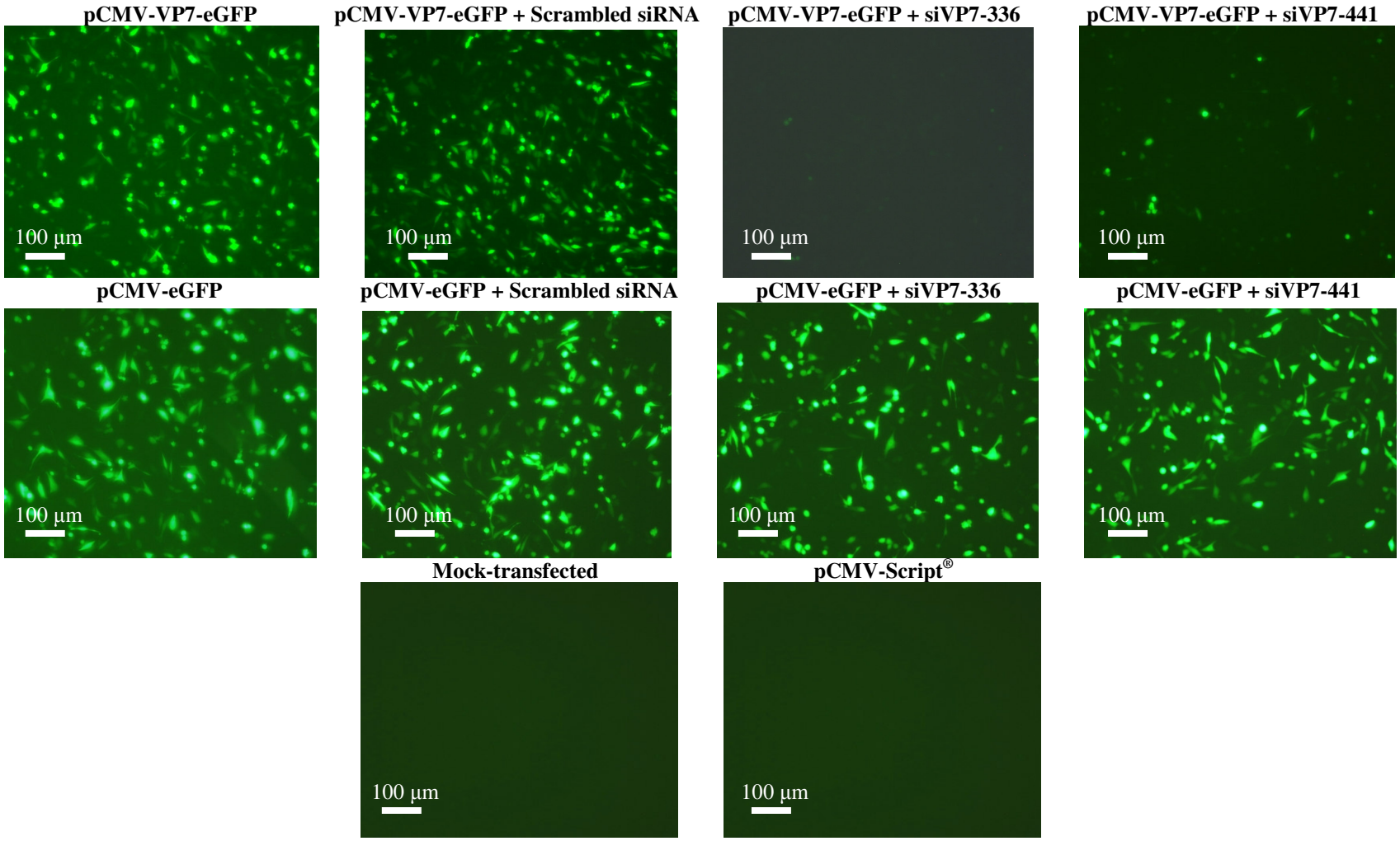

Fig. 3.7 **Inhibition of VP7-eGFP protein expression by VP7 gene-specific siRNAs in BHK-21 cells. BHK-21 cells were transfected with either pCMV-eGFP or pCMV-VP7-eGFP plasmid DNA and cotransfected with the VP7 gene-specific siRNAs, siVP7-336 and siVP7-441, as well as scrambled (negative control) siRNA. Mock-transfected BHK-21 cells and cells transfected with parental pCMV-Script® vector DNA are shown for comparative purposes. At 24 h post-transfection, representative fields were photographed.**

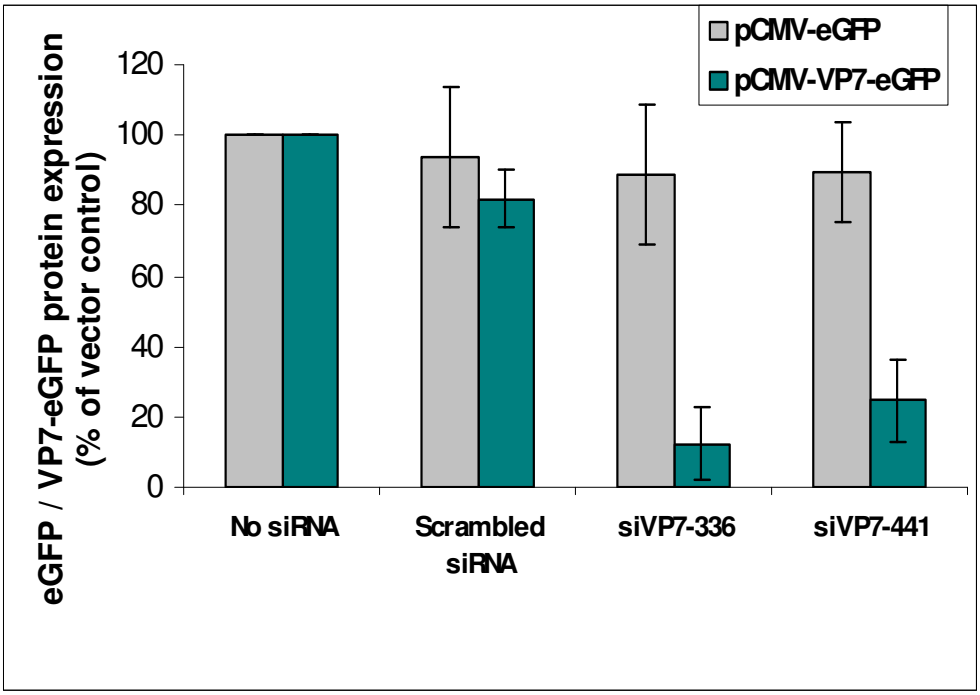

Fig. 3.8 **Silencing of VP7-eGFP expression by VP7-specific siRNAs in BHK-21 cells. BHK-21 cells were transfected with either pCMV-eGFP or pCMV-VP7-eGFP plasmid DNA and cotransfected with the VP7 gene-specific siRNAs siVP7-336 and siVP7-441, as well as scrambled (negative control) siRNA. At 24 h post-transfection, cells were analyzed for eGFP expression by fluorometry and expressed as percentages of the value for cells transfected with the recombinant expression vector constructs (no siRNA controls). The data are means ± standard deviations (SD) of values from five independent experiments.**

3.4 DISCUSSION

RNA interference (RNAi), a form of post-transcriptional gene silencing that results in the sequence-specific degradation of mRNA by homologous dsRNA molecules (Fire *et al*., 1998; Montgomery *et al*., 1998), has been used in several organisms including *C. elegans* (Fire *et al*., 1998; Montgomery *et al*., 1998), *Drosophila melanogaster* (Tuschl *et al*., 1999; Caplen *et al*., 2000), mosquitoes (Adelman *et al*., 2001; Caplen *et al*., 2002; Brown *et al*., 2003; Keene *et al*., 2004) and plants (Guo *et al*., 2003) to investigate the function of target genes. Although dsRNA induces the global shutdown of protein synthesis in mammalian cells (Manche *et al*., 1992; Williams, 1997; Stark *et al*., 1998), it is possible to suppress expression of endogenous and heterologous genes in mammalian cells by directly introducing siRNAs of less than 30 bp into the cells (Elbashir *et al*., 2001a; Caplen *et al*., 2001). Consequently, RNAi has become a powerful and preferred tool to study gene function in mammalian cell cultures.

In this part of the study, it was investigated whether AHSV-9 VP7 gene expression could be inhibited in mammalian (BHK-21) cells by using siRNAs targeted to different regions on the VP7 mRNA. Towards establishing such an assay, eGFP was initially used as reporter to assess the RNAi effect in BHK-21 cells and to optimize the assay parameters. To investigate, recombinant pCMV-Script® mammalian expression vectors containing the eGFP and a chimeric AHSV-9 VP7-eGFP gene were constructed, and expression of the eGFP and VP7-eGFP proteins in transfected BHK-21 cells was evidenced by brightly fluorescing cells. Expression of these proteins could not be confirmed by immunoblot analysis, probably as a consequence of the low levels of expression, and due to its insolubility, the highly hydrophobic VP7 protein could not be immunoprecipitated from radiolabelled cell lysates (results not shown). Thus, eGFP proved to be a sensitive reporter for VP7-eGFP protein expression in transfected BHK-21 cells. Following cotransfection of BHK-21 cells with the recombinant plasmid constructs and 20 pmol of siGFP-22, analysis of the results indicated that eGFP expression was suppressed by 95%, whilst expression of VP7-eGFP was suppressed to virtually background levels (97%) when compared to control cells transfected with the recombinant constructs only. These results are in agreement with the high level of siGFP-22-mediated inhibition of eGFP expression (90%) reported in primary mouse embryonic fibroblasts, HeLa and 293 cell cultures (Caplen *et al*., 2001). A scrambled (negative control) siRNA, however, suppressed VP7-eGFP gene expression by *ca.* 18 to 25%. Since this siRNA comprised a scrambled sequence of siGFP-22, it is possible that its use could have resulted in off-target effects, as the same siRNA inhibited eGFP expression by only 6%.

Since this study is the first to investigate whether RNAi can be applied to silencing expression of AHSV genes in mammalian cells, novel siRNAs targeted to the VP7 mRNA had to be designed. By taking a number of siRNA design recommendations into account (Elbashir *et al*., 2001a; 2001b; 2001c; Reynolds *et al*., 2004), two siRNAs, directed against different sites on the VP7 mRNA of AHSV-9, were designed and chemically synthesized. Care was taken especially to ensure that the siRNAs were completely complementary to the VP7 mRNA target sites and that they had very limited sequence homology to genes other than the intended target so as to avoid off-target effects. 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). The efficiency of the two siRNAs to mediate VP7-eGFP gene silencing was subsequently investigated and compared. BHK-21 cells were cotransfected with pCMV-VP7-eGFP and the siRNAs targeting different regions on the VP7 mRNA. Analysis of the results indicated that VP7-eGFP expression was specifically and significantly inhibited. Whereas siVP7-336 resulted in an 88% reduction in the expression of VP7-eGFP, siVP7-441 inhibited VP7-eGFP expression by 75%. These results are in agreement with several previous reports, indicating that siRNAs targeted to different sites on the same mRNA differ in their silencing efficiency (Harborth *et al*., 2001; Holen *et al*., 2002; Bohula *et al*., 2003; Vickers *et al*., 2003). Although complete inhibition of VP7-eGFP gene expression was not obtained and was indeed lower than that observed previously for siGFP-22 mediated silencing of eGFP and VP7-eGFP gene expression, the remaining activity might be explained by differences in transfection efficiency, insufficient internalization or both, and by differences in target site accessibility.

The above results indicated that siVP7-336 and siVP7-441 differed in their efficacy to silence VP7-eGFP gene expression. In at least five independent experiments, siVP7-336 consistently inhibited VP7-eGFP expression more efficiently than did siVP7-441. Therefore, the lower efficacy of siVP7-441 could not be attributed to differences in transfection efficiencies. At present, there is still a lack of clear understanding on the mechanisms that determine the gene-silencing efficiency of a given siRNA. It is, however, likely to involve both the primary sequence and secondary RNA structure, both in the siRNA and the targeted mRNA (Holen *et al*., 2002; Reynolds *et al*., 2004). Since siRNA-mediated gene silencing is regarded to be highly sequence-specific, it is reasonable to assume that a recognition step occurs between the siRNA and its target sequence. Indeed, Brown *et al*. (2005) recently provided evidence that target site access is directly linked to RISC catalysis. It therefore follows that the local target RNA segment has to be accessible to intermolecular nucleotide-nucleotide interactions with the siRNA. With reference to the VP7-eGFP RNA structure, the mRNA targeted by siVP7-336 had a small hairpin structure, whilst a larger hairpin

structure was seen in the mRNA targeted by siVP7-441. However, the latter was located in a region on the RNA that contains a number of similar structures in close proximity (Fig. 3.2). Based on the differences in local secondary structure, it may be possible that binding of the RISC/siVP7-446 complex with the target mRNA was sterically hindered by these multiple hairpin structures, thus resulting in less effective binding and consequently, less efficient degradation of the target mRNA. The higher efficiency of siVP-336 in suppressing VP7-eGFP expression is therefore consistent with observations that differences in the local structure of the targeted mRNA may affect the accessibility of the siRNA and hence, gene-silencing efficiencies (Bohula *et al*., 2003; Kretschmer-Kazemi Far and Sczakiel, 2003; Vickers *et al*., 2003; Reynolds *et al.*, 2004).

In conclusion, the results obtained in this part of the study supported previous findings that siRNAs could induce potent and specific gene silencing of heterologous genes in mammalian cells. Specifically, it was demonstrated that expression of eGFP and chimeric AHSV-9 VP7-eGFP transgenes are silenced effectively in BHK-21 cells. It was furthermore shown that properly designed siRNAs could effectively and specifically silence AHSV-9 VP7-eGFP expression, albeit that the siRNAs, targeting different regions on the VP7 mRNA, differed in their gene silencing efficiency. The availability of these siRNAs, coupled with the high level of specific gene silencing obtained, suggested that they may be potential therapeutic tools to inhibit AHSV-9 replication in infected cells. The details of these investigations are provided in the following Chapter.

CHAPTER FOUR

INHIBITION OF AFRICAN HORSESICKNESS VIRUS INFECTION IN BHK-21 CELLS BY RNA INTERFERENCE

4.1 INTRODUCTION

African horsesickness (AHS) is an infectious, non-contagious, arthropod-borne disease of the equids. The aetiological agent, African horsesickness virus (AHSV), belongs to the *Orbivirus* genus within the family *Reoviridae* (Calisher and Mertens, 1998). In susceptible horses the mortality rate post-infection can be as high as 95% (Coetzer and Erasmus, 1994). The disease is enzootic in sub-Saharan Africa, although outbreaks, resulting in considerable economic loss to the equestrian industry, have occurred in Morocco, the Middle East and in Europe (Howell, 1960; Mirchamsy and Hazrati, 1973; Lubroth, 1988; Brown and Dardiri, 1992). As a consequence of its severity and its ability to expand rapidly out of its endemic areas, AHS has been allocated OIE (Office International des Epizooties) List A status. The control of AHS in southern Africa is primarily based upon vaccination using a live-attenuated polyvalent vaccine (Erasmus, 1976; Taylor *et al*., 1992; Mellor and Hamblin, 2004), surveillance and strict zoning measures (Bosman *et al.*, 1995). Notably, the use of a live-attenuated vaccine has a number of shortcomings, most importantly, the possibility that attenuated strains may revert back to virulence and that reassortment of the AHSV genome segments between the live vaccine viruses and circulating field strain viruses, in either the host or *Culicoides* vector, may result in viruses with different or enhanced virulence characteristics (Mellor and Hamblin**,** 2004). It is therefore important to investigate alternate approaches for control of AHS.

RNA interference (RNAi) is an emerging technology that specifically inhibits gene expression. Small interfering RNAs (siRNAs), mediators of RNAi, are short (21-23 nt) RNA duplexes that inhibit gene expression by inducing sequence-specific degradation of homologous RNA (Fire *et al*., 1998; Montgomery *et al*., 1998; Tuschl *et al*., 1999; Elbashir *et al*., 2001b). Based on initial reports demonstrating that siRNA can significantly suppress viral gene expression when delivered into mammalian cells (Bitko and Barik, 2001; Gitlin *et al*., 2002), it has been proposed that RNAi may provide a new therapeutic approach for controlling viral infections. Subsequently, a number of groups have reported that siRNA could successfully inhibit replication of, amongst other, severe acute respiratory syndrome-associated coronavirus (Wang *et al.*, 2004; Zhang *et al.*, 2004b), human immunodeficiency virus-1 (Hu *et al.*, 2002), hepatitis C virus (Kapadia *et al*., 2003), poliovirus (Gitlin *et al*., 2002), west nile virus (McCown *et al.*, 2003), influenza A virus (Ge *et al*., 2003), rotavirus (Déctor *et al*., 2002), rous sarcoma virus (Jacque *et al.*, 2002), foot-and-mouth disease virus (Chen *et al.*, 2004; Liu *et al.*, 2005) and hepatitis B virus (McCaffrey *et al*., 2003).

Whereas most of the above studies have used siRNAs to block replication or to inhibit transcription of the viral genomes, an alternative strategy whereby viral infections may be controlled comprises

using siRNAs to hinder the proper assembly of infectious virus particles. Capsid protein-encoding genes should be favorable targets for degradation, since capsid proteins have essential functions in the virus life-cycle such as mediating attachment of virus particles to cell receptors, facilitating penetration of virions into the cell, and they are furthermore required for the assembly of virus particles prior to virus release (Levy *et al*., 1994). In the case of rotavirus, a non-enveloped double-stranded (ds)RNA virus from the family *Reoviridae*, it has been reported that siRNA targeting the VP4 mRNA not only inhibited expression of the VP4 capsid protein efficiently, but the yield of progeny was reduced. Furthermore, most of the particles purified from these cells were poorly infectious (Déctor *et al.*, 2002). In addition to rotavirus, RNAi has been used successfully to prevent replication of different picornaviruses in mammalian cell culture and these siRNAs have been directed mostly against the capsid-encoding regions in the viral genome. Gitlin *et al*. (2002) reported that RNAi prevented poliovirus infection in mammalian cells by using siRNAs against the P1-encoding region, which encodes the viral capsid proteins VP1 through VP4. Subsequently, they indicated that pretreatment of human and murine cells with siRNA duplexes directed to the capsid- and 3D polymerase-encoding regions resulted in a 100-fold reduction of the progeny titre. Similarly, Liu *et al*. (2005) reported that siRNA directed to the capsid protein VP4-encoding region of foot-and-mouth disease virus (FMDV) gave an inhibition of 50-fold in virus yield. These studies serve to confirm that the use of siRNAs targeting viral capsid protein genes may represent an effective antiviral strategy whereby viral infection can be prevented.

Like bluetongue virus (BTV), the prototype orbivirus, the AHSV virion is composed of seven structural proteins organized into a double-layered capsid containing the ten dsRNA segments of the viral genome (Verwoerd *et al*., 1972; Bremer, 1976; Huismans, 1979; Bremer, 1990; Huismans and van Dijk, 1990). When dsRNA is released into the cytoplasm of a mammalian cell, it elicits host cell defense mechanisms that can modify host cell translation control and lead to an apoptotic response. Presumably, to avoid shutdown of the host cell, the replication strategy of orbiviruses is such that only capped mRNA, which is produced internally within the core particle, is released into the cytoplasm of the infected cell (Jacobs and Langland, 1996; Mertens and Diprose, 2004). Thus, it may not be possible to inhibit transcription of orbiviral genes nor to block orbivirus genome replication using RNAi. However, based on cryoelectron microscopy of baculovirus-expressed BTV core-like particles and virus-like particles, as well as X-ray crystallography of the BTV core (Hewat *et al*., 1992a; Prasad *et al* 1992; Roy *et al*., 1997; Grimes *et al*., 1998), it has been hypothesized that the process of virion assembly may start with a nucleocapsid complex that is subsequently encapsidated by 60 dimers of VP3 molecules. Either shortly after, or simultaneously, 260 trimers of VP7 (organized in the icosahedron as $T = 13$) are added to the icosahedral scaffold of

VP3. At a later stage of virus morphogenesis the outer capsid proteins are added onto the surface of the core, possibly by interacting with VP7 and each other, and comprises 180 copies of VP2 and 120 trimers of VP5 (Hewat *et al*., 1992a; Stuart *et al*., 1998). It therefore follows that by silencing expression of VP7 it may be possible to abrogate or, at least, severely impair the formation of virus particles. Consequently, as VP2 and VP5 are involved in host cell attachment and penetration during the initial stages of infection (Hassan and Roy, 1999; Martinez-Torrecuadrada *et al*., 1999; Hassan *et al*., 2001), it may be that defective particle assembly could lead to non-infectious virus progeny and thus prevent virus infection.

In the previous chapter, it has been demonstrated that expression of the VP7 protein of AHSV-9 could be silenced effectively in mammalian (BHK-21) cells using siRNAs targeted against two different regions on the VP7 mRNA. Based on the important structural role that VP7 plays in the assembly of AHSV particles and on the premise that inhibition of its expression may impair formation of infectious virus particles, the primary aim of this part of the study was to investigate whether these VP7 gene-specific siRNAs could inhibit AHSV-9 infection in BHK-21 cells.

4.2 MATERIALS AND METHODS

4.2.1 Cell culture and viruses

Baby hamster kidney-21 (BHK-21) cells were propagated and maintained as monolayers in 75-cm^2 tissue culture flasks, and cultured in Minimum Essential Medium (MEM) Eagles base (Highveld Biological) supplemented with 2.5% or 5% (v/v) foetal bovine serum (FBS) and antibiotics (1 × penicillin, streptomycin, fungizone). The flasks were incubated at 37˚C in a humidified incubator with a constant supply of 5% CO_2. African horsesickness virus serotype 9 (AHSV-9), used in viral challenge assays, was kindly provided by Mr. F. Wege (Department of Genetics, University of Pretoria). AHSV-9 was propagated in confluent BHK-21 monolayers using a low-passage stock virus as inoculum according to previously described procedures (Huismans, 1979).

4.2.2 Viral challenge assay in BHK-21 cells

4.2.2.1 Small interfering RNAs (siRNAs)

The specificity and sequence of the siRNAs siVP7-336 and siVP7-441, targeting two different regions on the AHSV-9 VP7 mRNA, have been described previously (Chapter 3). Due to off-target effects observed by making used of a scrambled (negative control) siRNA, a universal negative siRNA was obtained from Qiagen. The siRNA, designated siUN-19, which reportedly lacks homology to known viral and cellular genes, has the following sequence: siUN-19, sense 5'-UUCUCCGAACGUGUCACGUdTT-3', antisense 5'-

ACGUGACACGUUCGGAGAAdTT-3'. All siRNAs were supplied by Qiagen as lyophilized, desalted duplexes and were suspended in an RNase-free buffer at a concentration of 20 µM prior to storage at -20˚C.

4.2.2.2 Transfection with siRNA and AHSV-9 infection

The day before transfection, cells were trypsinized, diluted with fresh medium and seeded in 35-mm-diameter wells to reach 80% confluency (*ca*. 9.6 × 10^5 cells/well) within 24 h of incubation at 37˚C in the presence of 5% CO_2. The BHK-21 cells were then transfected with the respective siRNAs using Lipofectamine™ 2000 according to the procedures described by Bitko and Barik (2001) and those in Chapter 3 (Section 3.2.5). Briefly, 5 µl of Lipofectamine™ 2000 reagent was diluted in 250 µl MEM medium (without serum and antibiotics) and, following incubation at room temperature for 5 min, was added to 250 µl antibiotic-and serum-free MEM medium containing either 100 or 200 pmol of the appropriate siRNA. The lipofection mixture (500 µl) was then incubated at room temperature for 20 min to allow formation of RNA-lipofectamine complexes. The BHK-21 cell monolayers were rinsed three times with 2 ml MEM medium (without serum and antibiotics) and then overlaid with the RNA-lipofectamine complexes. Following addition of 1.5 ml of antibiotic-free MEM medium containing 2.5% (v/v) FBS, the tissue culture dishes were incubated for 8 h at 37˚C in a CO_2 incubator. The transfected cells were then infected with AHSV-9 at a MOI of 1 p.f.u./cell. A second transfection was performed after 1 h of infection, as described above. At 24 h post-infection, cells were processed for RNA isolation, whilst the virus-containing supernatants were titrated in Vero cells.

4.2.3 Titration of viral progeny

The virus titre was determined in Vero cells, using the method described by Oellerman (1970). Cells infected with serial 10-fold dilutions of virus were overlaid with 4 ml of a sterile solution containing 0.5% (w/v) agarose, 30% Earles medium and 70% Eagles medium, and then incubated at 37˚C in a CO_2 incubator for 96 h. Plaques were visualized by the addition of Neutral Red (0.05% [w/v] in 1 × PBS), followed by incubation for 24 to 48 h, under the same conditions, until colorless plaques were visible against a red background.

4.2.4 Quantitative reverse transcription-polymerase chain reaction (RT-PCR)

4.2.4.1 Oligonucleotides

Quantification of specific mRNA by RT-PCR was performed using oligonucleotides designed based on AHSV-9 VP7 (GenBank acc. no. U90337), β2-microglobulin (β2-MG; GenBank acc. no. X17002) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; GenBank acc. no. U10983) sequences, and are indicated in Table 4.1. The RT-PCR amplicons were 135, 138 and 104 bp in size, respectively, for these mRNAs. The oligonucleotides were designed using Primer3 (Rosen and Skaletsky, 2000) and DNAMAN v.4.13 (Lynnon Biosoft), whilst optimal oligonucleotide pairs were analyzed by PerlPrimer v.1.1.6

(Marshall, 2004). BLASTN (available at http://www.ncbi.nlm.nih.gov/BLAST) was used to verify the target sequence specificity of each newly designed oligonucleotide pair.

Table 4.1 Oligonucleotides used in quantitative real-time RT-PCR

Oligonucleotide	Nucleotide sequence	Tm (°C)	Annealing position	Target mRNA
GAPDH-F	5' - GCATGGCCTTCCGTGTTCCTAC - 3'	67.1	653-674	BHK GAPDH*
GAPDH-R	5' - CCTGCTTCACCACCTTCTTGATGTC - 3'	67.3	733-757	BHK GAPDH
b-2 MG-F	5' - AGTGGAGCTGTCAGATCTGTCCTTC - 3'	64.5	9-34	BHK β2-MG#
b-2 MG-R	5' - TGACCACCTTGGGCTCCTTC - 3'	64.8	127-147	BHK β2-MG
VP7-F9	5' - CTGGAGATGTCGTCGCATGGAATAC - 3'	67.9	656-680	AHSV-9 VP7
VP7-R9	5' - GAGCCAATTCCGGAACCGTG - 3'	67.1	774-793	AHSV-9 VP7

*GAPDH glyceraldehyde-3-phosphate dehydrogenase
*β2-MG β2-microglobulin

4.2.4.2 RNA extraction

For detection of the targeted gene expression in BHK-21 cells at 24 h post-infection, total RNA was extracted from BHK-21 cell cultures, using the Aurum™ Total RNA extraction kit (BioRad) according to the manufacturer's instructions. The culture medium was aspirated and the cells were rinsed once with 1 × 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), trypsinized and then collected by centrifugation at 3 000 rpm for 10 min. The cell pellets were suspended in 350 µl of the supplied lysis solution, lysed by vigorous pipetting (at least 12 times) and then transferred to a 2-ml Eppendorf tube. Following addition of an equal volume of 70% ethanol, the cell lysate was centrifuged through a RNA-binding column at 15 000 rpm for 30 s, after which the column was rinsed with wash solution, treated with DNase I to remove contaminating genomic DNA (15 min at room temperature) and the RNA was then eluted from the column using the supplied elution buffer. To confirm the absence of contaminating DNA, aliquots of the RNA preparation were used in PCR reactions together with the oligonucleotide pairs indicated in Table 4.1. The PCR reactions were performed, as described previously (Section 2.2.3.2), except that annealing of the oligonucleotides was performed at 60˚C for 30 s. Following PCR, aliquots of each reaction mixture were analyzed by electrophoresis on 0.8% (w/v) agarose gels in the presence of appropriate DNA molecular weight markers (Fermentas).

4.2.4.3 cDNA synthesis

The RNA was reverse transcribed using the QuantiTect® Reverse Transcription kit (Qiagen) according to the manufacturer's instructions. Prior to reverse transcription, each RNA preparation (10 pg-1 µg) was incubated at 42˚C for 2 min with 1 × genomic DNA wipeout buffer (supplied in the kit) to ensure complete removal of contaminating genomic DNA. To reverse transcribe the RNA, 4 µl of 5 × Quantiscript® RT buffer, 1 µl Quantiscript® Reverse Transcriptase and 1 µl of RT primer mix, which contains an optimized

blend of oligo-dT and random primers, were added to the RNA preparation in a final volume of 20 µl. Reverse transcription was performed at 42˚C for 30 min, after which the enzyme was inactivated by heating to 95˚C for 3 min and the reaction mixtures stored at -20˚C.

4.2.4.4 Quantitative real-time PCR

Quantitative real-time PCR was performed using the QuantiTect™ SYBR® Green PCR kit (Qiagen) and the LightCycler® 1 system (Roche). Each reaction mixture (20 µl) contained 1 µl of the reverse transcription reaction mixture, 10 pmol of gene-specific sense and antisense oligonucleotides and 10 µl of 2 × QuantiTect™ SYBR® Green PCR master mix (consisting of HotStar*Taq*® DNA polymerase, dNTP mixture inclusive of dUTP, SYBR® Green I, ROX passive reference dye and 5 mM $MgCl_2$). The reaction mixtures were incubated at 95˚C for 15 min to activate the HotStar *Taq*® DNA polymerase enzyme and then subjected to 55 cycles of denaturation at 94˚C for 15 s, oligonucleotide annealing at 60˚C for 20 s and extension at 72˚C for 10 s. For each target gene, reaction mixtures identical to those described above were prepared, except template was omitted. Aliquots of each reaction mixture were analyzed by agarose gel electrophoresis in the presence of appropriate DNA molecular weight markers. During each cycle, data acquisition was performed during the extension step and analyzed using LightCycler® software v.3.5.3 (Rasmussen, 2001). To compare the amplification efficiencies of different target sequences, a dilution series of each target was prepared in triplicate and each dilution series was amplified. To confirm the specific amplification, melt-curve analysis of the amplicons was performed by decreasing the temperature from 95˚C to 65˚C with a temperature transition rate of 20˚C/s and then increasing the temperature to 95˚C at a rate of 0.1˚C/s with continuous fluorescence measurement. For Crossing Point (CP) determination, the Second Derivate Maximum Method was used, which is included in the LightCycler® software package.

4.2.5 Statistical analyses

The *BestKeeper* software tool (Pfaffl *et al*., 2004) was used to determine the stability of the two housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β2-microglobulin (β2-MG). Group-wise comparison and statistical analysis of the relative expression results were performed, using the Relative Expression Software Tool (REST©) (Pfaffl *et al*., 2002). The relative expression of AHSV-9 VP7 mRNA in each sample was computed using the REST© software tool, based on its real-time PCR efficiencies (*E*) and the crossing point (CP) difference of a VP7-specific siRNA-treated sample versus an universal negative siRNA-control sample. Based on the CP values, the relative expression of AHSV-9 VP7 mRNA in each sample was obtained by normalizing to β2-MG mRNA expression levels, and then the percentages of AHSV-9 VP7 mRNA in VP7-specific siRNA-transfected cells relative to that in universal negative siRNA-transfected cells was calculated.

4.3 RESULTS

4.3.1 Selection of an appropriate reference gene for quantitative real-time PCR

Quantitative real-time PCR has become the favored tool in mRNA expression analysis (Mackay *et al.*, 2002; Ding and Cantor, 2004). However, the most prominent problem in quantitative mRNA expression analysis is the selection of an appropriate reference gene. The use of such genes relies on the premise that they exhibit a constant basal level of expression that is consistent, non-regulated and independent of cell cycle (Thellin *et al.*, 1999; Suzuki *et al.*, 2000). One of the most widely used reference genes in RNAi assays is the β-actin gene (Weisinger *et al.*, 1999). However, there is a growing body of evidence suggesting that β-actin is an unsuitable control in quantitative mRNA expression analysis due to setting-dependent variations in its expression (Chang *et al.*, 1998; Foss *et al.*, 1998; Schmittgen and Zakrajsek, 2000; Selvey *et al.*, 2001; Radonić *et al.*, 2004). In a recent study, ten different potential reference genes were compared for their use in experiments investigating cellular mRNA expression of virus-infected human cell lines (Radonić *et al.*, 2005). Whereas glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β2-microglobulin (β2-MG) showed stable expression in virus-infected cells, β-actin showed significant variations with an increasing degree of infection, and all the other genes showed moderate expression stability. Consequently, the GAPDH and β2-MG housekeeping genes were selected and their suitability as reference genes for use in subsequent quantitative real-time PCR analysis evaluated.

To investigate, BHK-21 cell monolayers were transfected with 100 pmol of a negative control siRNA, the VP7 gene-specific siRNAs siVP7-336 and siVP7-441 or a cocktail thereof (100 pmol of each siRNA). At 8 h post-transfection the cells were infected with AHSV-9, after which the cells were transfected a second time with the siRNAs, as described above. As a control, cells infected with AHSV-9 and mock-infected cells were included in the analysis. At 24 h post-infection the cells were harvested, total RNA extracted and treated extensively with DNase prior to performing reverse transcription and quantitative real-time PCR. The data was analyzed using the *BestKeeper* software tool (Pfaffl *et al.*, 2002) in order to identify the most suitable reference gene in this experimental setting.

Using the *BestKeeper* software tool, descriptive statistics of the derived crossing points (CP), generated by the real-time PCR platform, were computed for both the GAPDH and β2-MG housekeeping genes. All CP data were compared over the entire study, comprising of the mock-transfected cells, AHSV-9-infected cells, siUN-19-treated virus-infected cells and all test groups (VP7-specific siRNA-treated virus-infected cells). According to the variability observed,

housekeeping genes can be ordered from the most stably expressed, exhibiting the lowest variation, to the least stable one, exhibiting the highest variation. Any studied gene with the standard deviation (SD) higher than 1 can be considered inconsistent and should thus not be used as a reference gene (Pfaffl *et al.*, 2004). In this regard, β2-MG had a standard deviation below 1 (SD = 0.85), whereas GAPDH had a standard deviation above 1 (SD = 1.08). β2-MG also exhibited a lower crossing point variation (CV[%CP] = 2.73) compared to GAPDH (CV[%CP] = 4.36), and is therefore considered to be the most stably expressed and most reliable housekeeping gene in this experimental setting. The descriptive statistics, generated by the *BestKeeper* software tool for both housekeeping genes, are indicated below in Table 4.2.

Table 4.2 Descriptive statistics of β2-microglobulin (β2-MG) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping genes based on their crossing point (CP) values

Factor	β2-MG	GAPDH
n	8	8
GM [CP]	31.00	24.69
AM [CP)]	31.03	24.74
Min [CP]	27.87	21.22
Max [CP]	32.89	27.08
SD [±CP]	0.85	1.08
CV [% CP]	2.73	4.36

Abbreviations: n: number of samples; CP: the crossing points generated by the real-time PCR platform; GM [CP]: the geometric mean of CP; AM [CP]: the arithmetic mean of CP; Min [CP] and Max [CP]: the extreme values of CP; SD [± CP]: the standard deviation of the CP; CV [%CP]: the coefficient of variance expressed as a percentage on the CP level.

Based on the descriptive statistics generated by the *BestKeeper* software tool, the β2-MG gene was thus selected as the most suitable reference gene since it was stably expressed between the control and tests samples. Therefore, the amount of AHSV-9 VP7 mRNA in each of the samples was subsequently determined by normalizing to that of β2-MG.

4.3.2 Reduction of AHSV-9 VP7 mRNA by VP7 gene-specific siRNAs in BHK-21 cells

It is thought that siRNAs target mRNAs containing homologous sequences and induce their cleavage. Thus, the effects of siVP7-336 and siVP7-441 on the abundance of AHSV-9 VP7 mRNA in virus-infected BHK-21 cells were investigated. BHK-21 cells were transfected with 100 pmol of siVP7-336 or siVP7-441, a cocktail comprising 100 pmol of each siRNA or with 200 pmol of siVP7-336. Cells transfected with negative control siRNA (siUN-19) were included as controls. After incubation for 8 h, the transfected cells were infected with AHSV-9 at a MOI of 1 and the cells were again transfected 1 h post-infection with the siRNAs, as described above. Total RNA

was isolated from cells 24 h post-infection, treated extensively with DNase I and subjected to reverse transcription followed by relative quantitative PCR. The amount of VP7 mRNA was normalized to that of β2-MG mRNA in the sample.

The data, presented in Fig. 4.1, indicated that both siVP7-336 and siVP7-441 reduced the amount of VP7 mRNA in the AHSV-9-infected cells. As expected, siVP7-336 was more effective in inhibiting VP7 mRNA (93%) compared to siVP7-441 (86%). These results correlated well with the results obtained previously, confirming the difference in efficiency of inhibition between these siRNA molecules (Chapter 3, Fig. 3.8). By increasing the dosage of siVP-336 (200 pmol pre- and post-infection) or by making use of a cocktail of the two VP7 gene-specific siRNAs, the amount of VP7 mRNA was reduced by 81% and 89%, respectively.

The possibility of DNA contamination in the RNA preparations used above was eliminated by performing a DNase treatment and verified by subjecting the samples to PCR amplification, using *Taq* DNA polymerase and the gene-specific oligonucleotide pairs. No amplicons were obtained from control reaction mixtures lacking template or from the RNA samples used as templates for quantitative real-time PCR following reverse transcription. In addition, the amplification specificity of the quantitative real-time PCR was verified by agarose gel electrophoresis and a single amplicon of the expected length for each target was obtained (VP7, 135 bp; β2-MG, 138 bp) (results not shown). Furthermore, to confirm its reproducibility, melt curve analysis was performed and the efficiency of the quantitative real-time PCR was calculated. Serial dilutions, in triplicate, of the cDNA prepared from mock-transfected cells, siRNA-treated and AHSV-9 infected cells, and virus-infected cells were subjected to quantitative real-time PCR. Using the REST© software tool (Pfaffl *et al*., 2002), the data obtained indicated that the calculated PCR efficiencies (*E*) for both targets (E_{VP7} =1.89 and $E_{B2\text{-}MG}$ = 1.93) were close to the theoretic maximum and optimum efficiency of *E* = 2.0, thus indicating that the quantitative real-time PCR had a high level of reproducibility and sensitivity (Table 4.3). The amplification plots and melting curves of VP7 and β2-microglobulin amplicons are provided in the Appendix to this dissertation.

Based on all of the above, the data therefore indicate that siVP7-336 and siVP7-441 reduced the accumulation of VP7 mRNA in AHSV-9 infected BHK-21 cells, whilst it had no effect on β2-MG mRNA.

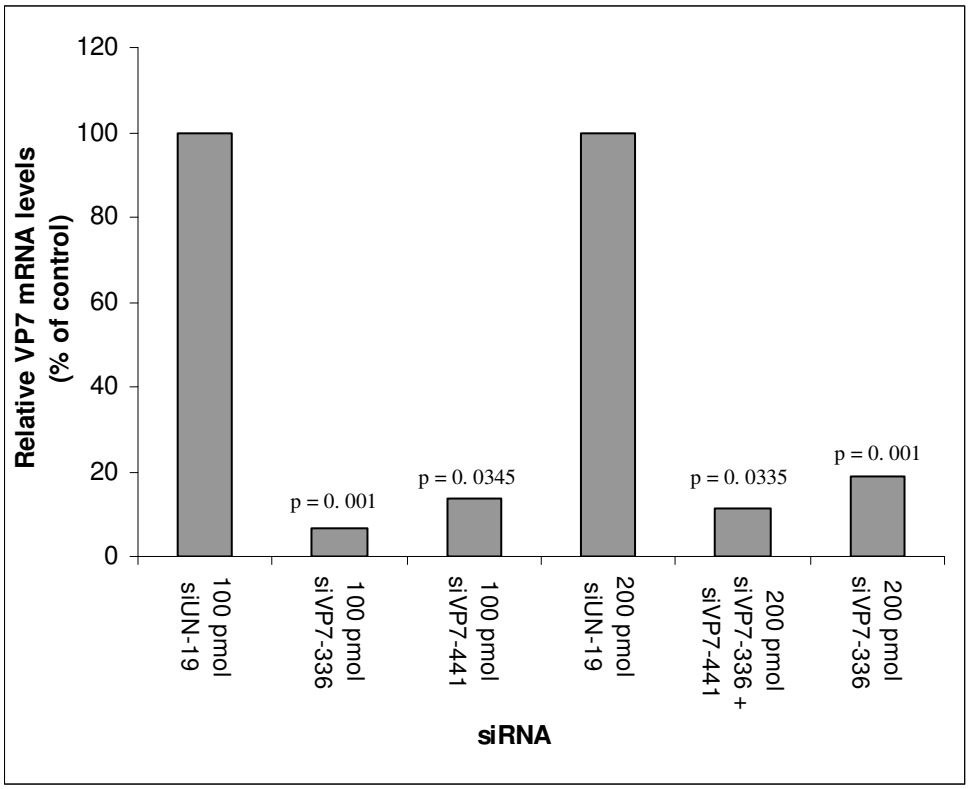

Fig. 4.1 **Inhibition of AHSV-9 VP7 mRNA by siVP7-336 and siVP7-441 in AHSV-9-infected BHK-21 cells. Total RNA isolated from siRNA-treated AHSV-9-infected cells 24 h post-infection were subjected to quantitative real-time PCR analysis of VP7 mRNA. The data were normalized to the amount of β2-microglobulin mRNA and are expressed as percentages of the normalized value for AHSV-9-infected cells transfected with the siUN-19 (negative control) siRNA. The precision and reproducibility of the real-time PCR was confirmed by three replicates of each sample. P-values ≤ 0.01indicate a significant difference between the VP7 mRNA levels in cells transfected with VP7-specific siRNA versus the VP7 mRNA levels in siUN-19-transfected cells.**

Table 4.3 Reproducibility of quantitative real-time PCR

siRNA treatment of BHK-21 cells*	Threshold cycle (C_T) values (of three replicates)		Mean C_T values	
	β2-MG	VP7	β2-MG	VP7
Mock-transfected	27.68 27.82 28.10	- - -	27.867	-
AHSV-9 infected	30.91 31.07 31.45	21.29 21.43 21.57	31.143	21.430
Transfected with 100 pmol of universal negative siRNA; AHSV-9 infected	31.15 31.29 31.19	20.60 20.55 20.77	31.21	20.64
Transfected with 100 pmol of siVP7-336; AHSV-9 infected	30.78 30.80 30.85	24.30 24.43 24.28	30.810	24.337
Transfected with 100 pmol of siVP7-441; AHSV-9 infected	31.18 31.14 30.91	23.47 23.55 23.51	31.077	23.51
Transfected with 200 pmol of universal negative siRNA; AHSV-9 infected	32.17 32.28 31.88	21.85 21.83 21.91	32.11	21.863
Transfected with cocktail of 200 pmol each of siVP7-336 and siVP7-441; AHSV-9 infected	30.85 31.03 30.90	24.04 23.98 23.97	30.927	23.997
Transfected with 200 pmol of siVP7-336; AHSV-9 infected	32.93 32.92 32.83	25.08 25.16 25.26	32.893	25.167

* Dosages of siRNAs indicate those used pre- and post-infection

4.3.3 Inhibition of AHSV-9 virus production by VP7 gene-specific siRNAs in BHK-21 cells

It has been reported that the inhibition of viral capsid proteins by siRNAs can interfere with the assembly of viral particles during morphogenesis, thereby resulting in a reduction in the number of infectious particles released from infected cells (Déctor *et al.*, 2002; Gitlin *et al.*, 2002; Liu *et al.*, 2005). Since AHSV-9 can replicate rapidly in infected cells (Huismans, 1979), the ability of the VP7 gene-specific siRNAs to likewise inhibit virus production was tested. After 24 h of culture, the virus in the medium of the cells should represent newly replicated virus in the cells. Thus, to validate the siRNA effects on replication of AHSV-9, virus titrations were performed 24 h post-infection by assaying the extracellular virus in BHK-21 cells that had been transfected with the siRNAs and then infected with AHSV-9, as described previously.

The results obtained indicated that the siUN-19 (negative control)-transfected cells did not exhibit a reduction virus titre compared to AHSV-9-infected BHK-21 cells, thus suggesting that the virus replicated in the cells during the 24-h culture period. In contrast, the virus titre was decreased in the samples where the cells were transfected with VP7 gene-specific siRNAs prior to and following virus infection, demonstrating that these siRNAs specifically inhibited AHSV infection. In agreement with its ability to silence VP7 protein and mRNA expression effectively, siVP7-336 was found to inhibit virus propagation the most effectively and reduced the infectious viral titres by 84%. Likewise, in concordance with its VP7 silencing profile, siVP7-441 was less effective and induced up to 63% reduction in virus plaque formation. In BHK-21 cells treated with either siVP7-336 at higher dosages or a mixture of siVP7-336 and siVP7-441, the infectious viral titres were reduced by 71% and 73%, respectively (Fig. 4.2).

From these results it was concluded that siVP7-336 and siVP7-441, albeit with different efficiencies, mediated a reduction in cytoplasmic VP7 mRNA levels and hence expressed VP7 protein, thus ultimately resulting in a decrease in the viral progeny released from infected cells. Notably, increasing the dosage of siVP7-336 or using a cocktail of the two VP7 gene-specific siRNAs did not result in increased inhibition of virus production. It is worth noting that, since not all cells can be transfected with the siRNAs, a number of them will sustain virus replication. In agreement with this, the titres were not zero but rather greatly reduced.

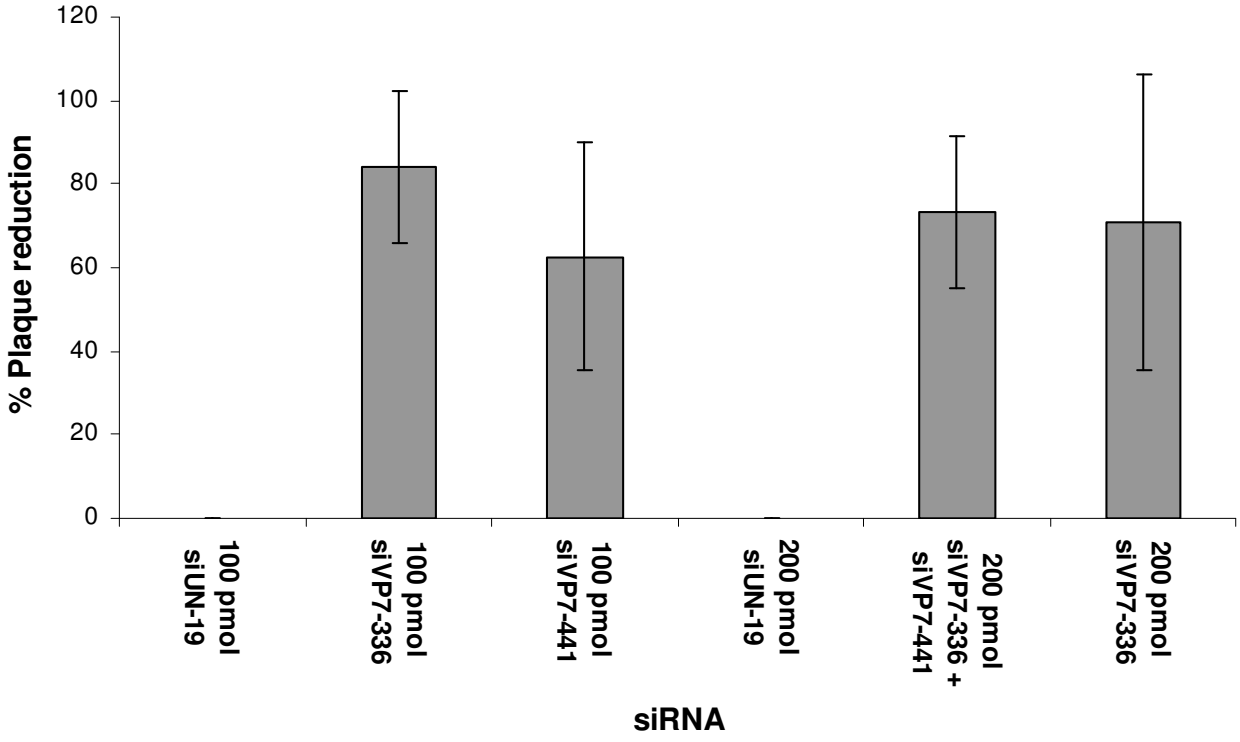

Fig. 4.2 **Inhibition of AHSV propagation in BHK-21 cells. The effect of the VP7 gene-specific siRNAs siVP7-336 and siVP7-441 on AHSV-9 progeny virus titres is depicted as percent reduction in plaque forming units, as compared to siUN-19 (negative control) siRNA at the same concentrations (with 0% reduction). The data are means ± standard deviations (SD) of values from two independent experiments.**

4.3 DISCUSSION

Post-transcriptional gene silencing (PTGS) mediated by siRNA has been a well-proven method for controlling replication of different viruses of plant origin (Lecellier and Vionnet, 2004). Initially there were apprehensions about a siRNA-mediated interference mechanism operating in mammalian systems owing to dsRNA-induced apoptosis, but Elbashir et al. (2001a) had proven that direct introduction of siRNAs can induce RNAi in mammalian cells. Subsequently, many mammalian viruses have been demonstrated to be amenable to siRNA-mediated suppression both in cell culture systems and in in vivo systems (Haasnoot et al., 2003; McCaffrey et al., 2003; Tan and Yin, 2004; Sanchez-Vargas et al., 2004; Tompkins et al., 2004). This method offers a promising alternative method for control of animal viruses, since foot-and-mouth disease (FMDV) virus has also been found to be responsive to siRNA (Chen et al., 2004; Liu et al., 2005). In this part of the study, RNAi was thus utilized to determine the efficiency of this technique for inhibition of AHSV-9 infection of mammalian (BHK-21) cells, using siRNAs that have been shown previously to silence VP7 protein expression.

To evaluate the effect of different siRNAs on AHSV-9 VP7 mRNA expression levels in infected cells, quantitative real-time RT-PCR was used in this study. As the validity of the conclusions drawn from such mRNA expression analysis is highly dependent on the reference gene used, the selection of an appropriate reference gene for data normalization warrants some discussion. In relative quantification, housekeeping genes have long been considered suitable as reference genes based on their reported stable expression (Suzuki et al., 2000). However, numerous studies have shown that expression of housekeeping genes can vary considerably, depending on the experimental conditions used (Chang et al., 1998; Thellin et al., 1999; Suzuki et al., 2000; Selvey et al., 2001; Radonić et al., 2005). Since an “ideal” reference gene does not appear to exist, it is therefore prudent to rather compare the gene expression of different housekeeping genes, under the experimental conditions to be used, in order to identify the most appropriate reference gene (i.e. most stably expressed). In this study, β2-microglobulin (β2-MG), a component of the major histocompatibility complex (MHC) class I molecules, and glyceraldehydes-3-phosphate dehydrogenase (GAPDH), a key enzyme in glycolysis, were selected since they are both stably expressed in virus-infected cells (Radonić et al., 2005). Of the two housekeeping genes, β2-MG was found to be the most stably expressed under the experimental conditions used in this study (Table 4.2) and was therefore selected for data normalization in quantitative real-time PCR analysis. The variation in GAPDH expression observed may be attributed to its functional diversity, which

includes, amongst other, roles in microtubule bundling, nuclear RNA export, and DNA replication and repair (Sirover, 1999).

Orbiviruses are highly lytic and have a rapid replication cycle. It has been reported that at 11 h post-infection, protein synthesis in BTV-infected cells is predominantly virus-specified due to the generation of progeny virus (Huismans, 1979). Consequently, the effects of different siRNAs on VP7 mRNA expression and viral infection in BHK-21 cells were evaluated 24 h post-infection of the siRNA-treated cells. Whereas the effect of the siRNAs on the abundance of VP7 mRNA in virus-infected cells was analyzed by quantitative RT-PCR analysis, the effect of siRNA-mediated inhibition of AHSV-9 infection was determined by performing plaque assays.

The results indicated that transfection of BHK-21 cells with 100 pmol of siVP7-336 and siVP7-441 prior to and following AHSV-9-infection reduced VP7 mRNA expression by 93% and 86%, respectively. The differences in efficacy may be ascribed to differences in the local secondary structure of the regions targeted by the respective siRNAs, since secondary structure analysis of the VP7 mRNA has indicated that the region targeted by siVP7-441 is structurally complex and could hinder accessibility to the target region (Fig. 3.2; Chapter 3). Nevertheless, the results obtained compare favorably with the level of inhibition of VP7-eGFP protein expression observed previously for these siRNAs (88% for siVP7-336 and 75% for siVP7-441; Chapter 3). Therefore, since the reduction in the amount of viral mRNAs was as large as the reduction in the amount of eGFP-VP7 protein, it is likely that the siRNA-induced silencing complex mediated sequence-specific VP7 mRNA degradation. The extent of inhibition of VP7 mRNA synthesis achieved with a combination of these siRNAs (89%) or by increasing the dose of siVP7-336 (81%) did not improve the inhibition from that achieved with siVP7-331 alone. The decrease in inhibition may be the result of a dose-inhibitory effect of the siRNA on the transfection reagent and could subsequently have resulted in a lower effective concentration in the intracellular siRNAs, or, alternatively, the reduction of the overall inhibition efficacy using both siRNAs compared to utilization of siVP7-336 alone could be due to competition from the less efficacious siVP7-441 to bind to its target sequence on the VP7 mRNA.

To test whether siRNAs inhibited AHSV production, culture supernatants were harvested at 24 h post-infection and the virus titre determined by plaque assays. Since the replication cycle is completed within 11 h post-infection (Huismans, 1979), the virus in the culture medium after 24 h of culture was considered to represent newly replicated virus. Compared to the titre obtained from AHSV-9 infected cells, a significant reduction in the virus titre was observed for BHK-21 cells

treated with siVP7-336 (84%), but an increase in dosage did not result in an improvement (71%). In agreement with its less efficacious silencing profile, siVP7-441 and a cocktail of both VP7 gene-specific siRNAs had a less remarkable effect (63% and 73%, respectively). Replication of AHSV-9 was, however, not affected in cells treated with a universal negative siRNA, indicating that the observed decrease in virus titre was not due to a cytotoxic effect of the siRNA duplexes or the result of a general antiviral response activated by the siRNAs. Since VP7 is an important structural determinant for viral assembly, especially at the level of capsid formation, the decrease in viral titre may be due to the formation of less infectious viral particles. The structural protein VP7 is a major component of the core particles and serves as an anchor for the outer capsid proteins VP2 and VP5 during virus morphogenesis (Hewat et al., 1992a; Stuart et al., 1998). Since both capsid proteins are essential for host cell attachment and penetration during the early stage of infection (Hassan and Roy, 1999; Martinez-Torrecuadrada et al., 1999; Hassan et al., 2001), it follows that virus particles formed in the absence of VP7 might be aberrant and therefore less infectious compared to those formed in its presence. The residual infectious virus produced in the presence of the siRNAs was most likely the result of virus replication in cells that were not transfected with the siRNAs.

The results above suggest that although the virus titres were reduced, most likely due to inhibition of VP7 mRNA and protein expression, AHSV replication, however, may not have been blocked. During infection, the core particles derived from the infecting viruses synthesize ten viral RNA transcripts that are translated. The lack of free pools of non-packaged viral dsRNA in infected cells has been taken to suggest that not only do the mRNA transcripts serve as templates for protein synthesis, but also that they are encapsidated, with the minus-strands subsequently being made using the packaged viral plus-strands as templates (Mertens and Diprose, 2004). Results obtained with rotavirus, another member of the Reoviridae family, have indicated that siRNAs directed to a given transcript impaired the synthesis of the corresponding protein, but not of the corresponding dsRNA segment. Consequently, rotavirus particles containing all 11 RNA segments in equimolar amounts are formed, despite the fact that synthesis of the target protein is almost completely ablated and the mRNA has been degraded (Déctor et al., 2002; Silvestri et al., 2004). It was proposed that viral plus-strand RNAs establish two, functionally different and physically separated pools. One pool is located outside of the viroplasms (the intracellular site of virus morphogenesis and replication), which undergo translation and are susceptible to siRNA-induced degradation, whereas the second pool is located inside viroplasms, which undergo replication and are inaccessible to the RNAi machinery. The formation of virus inclusion bodies (VIBs) during AHSV replication, combined with its highly lytic and rapid replication cycle (Huismans, 1979), may similarly present a challenge for the RNAi machinery to block the replication of the virus.

In conclusion, the results obtained in this part of the study indicated that siRNA corresponding to the AHSV VP7 gene sequence promoted ablation of the VP7 mRNA, resulting in the formation of less infectious AHSV particles. Although the use of RNAi as an antiviral therapeutic principle and method still remains to be demonstrated, inhibition of viral gene expression by siRNAs may offer the potential for a novel therapeutic approach for infections by AHSV, and possibly, other orbiviruses. Especially, the nonstructural protein NS2, which is responsible for the formation of VIBs in infected cells (Thomas et al., 1990; Brookes et al., 1993; Uitenweerde et al., 1995) and NS3, which is thought to be virulence determinant and plays a role in the release of virus particles from infected cells (Hyatt et al., 1993; van Staden et al., 1995; O'Hara et al., 1998), may be attractive targets for antiviral therapy. The "proof-of-principle" established in this study, should pave the way for such studies to be undertaken.

CHAPTER FIVE

CONCLUDING REMARKS

RNA interference (RNAi) has become a powerful and widely used tool for the analysis of gene functions. In mammalian cells, dsRNA fragments longer than 30 bp induce components of the interferon response, which mediate a generalized inhibition of gene expression through the non-specific shutdown of protein synthesis and mRNA degradation (Manche *et al*., 1992; Stark *et al.*, 1998). The demonstration that synthetic 21-nt siRNA duplexes effectively inhibit mammalian endogenous gene expression in a sequence-specific manner without activating the non-specific dsRNA responses (Elbashir *et al.*, 2001a; Caplen *et al.*, 2001) offers great opportunities to use RNAi to study the function of mammalian virus genes (Bitko and Barik, 2001; Colbère-Garapin *et al*., 2005; López *et al*., 2005), and it also offers great potential as an antiviral therapeutic agent (Gitlin and Andino, 2003; Tan and Yin, 2004). Consequently, the aims of this investigation were essentially to develop RNAi assays whereby expression of the VP7 gene of African horsesickness virus (AHSV) could be silenced in *Spodoptera frugiperda* insect cells and in mammalian BHK-21 cells, and to evaluate the efficacy of VP7 gene-specific siRNAs as a means to inhibit AHSV-9 infection in BHK-21 cells. The details of the results obtained in the course of achieving these objectives have been discussed in the individual chapters. The new information that has evolved during this investigation will be briefly summarized and suggestions regarding the future research in this field will be made.

The bacmid/insect cell expression system has greatly facilitated studies aimed at understanding the function of individual AHSV proteins (Martinez-Torrecuadrada *et al*., 1994; Maree and Huismans, 1997; Roy *et al*., 1997; van Staden *et al*., 1998; Kar *et al*., 2004) and the assembly of structural proteins into core- and virus-like particles through co-expression of the respective virus proteins (Roy *et al*., 1992; Roy *et al*., 1993; Roy *et al*., 1994a; Maree *et al*., 1998). Since RNAi represents a powerful tool to not only elucidate the function of different genes and encoded proteins, but also to explore different steps during the virus replication cycle, amongst other, particle assembly (López *et al*., 2005), the usefulness of RNAi as a means to silence transgene expression in *S. frugiperda* insect cells was investigated (Chapter 2). The results obtained indicated that eGFP production by a recombinant bacmid carrying the eGFP gene was inhibited by 75% after transfecting the cells with a cognate siRNA, siGFP-22. In the only other study of this nature, aminopeptidase N transgene expression was reported to be inhibited by 70% using long dsRNAs corresponding to the transgene (Agrawal *et al.*, 2004). Although insect cells lack an interferon response (Katze, 1995) and posses Dicer activity (Flores-Jasso *et al.*, 2004), a disadvantage of the latter approach is the possibility that non-specific gene silencing might occur owing to the presence of several siRNA molecules. Consequently, the methodology developed and described in this dissertation for siRNA-directed silencing of transgene expression in *S. frugiperda* cells represents an improvement of that published

previously. However, despite the comparatively higher level of inhibition obtained, it should be noted that the assay is laborious, costly and time-consuming, as it requires extensive optimization of several different parameters.

Based on reports demonstrating potent and specific siRNA-mediated inhibition of endogenous and heterologous gene expression in mammalian cells, it was next investigated whether expression of the AHSV-9 VP7 gene could be silenced in BHK-21 cells using VP7 gene-specific siRNAs. By contrast to enzymatically generated siRNAs (Donze and Picard, 2002; Yang *et al.*, 2002; Kawasaki *et al.*, 2003; Myers *et al.*, 2003) and siRNA-expressing plasmids or viral vectors (Brummelkamp *et al.*, 2002; Paddison *et al.*, 2002a; 2002b; Rubinson *et al.*, 2003; Shen *et al.*, 2003; Schuck *et al.*, 2004), chemically synthesized siRNA, which are of uniform composition, represents the "gold standard" for RNAi applications. Despite being the most expensive way to produce siRNAs, chemically synthesized siRNA directed at a single mRNA target is, however, the least likely to produce off-target effects. Consequently, in this investigation, chemically synthesized siRNAs were chosen as the preferred means to mediate sequence-specific silencing of the AHSV-9 VP7 gene. Due to the sequence-dependent variability of siRNA efficacy, the design of effective siRNA is an important factor to consider. On the basis of analyses of a number of target genes that were successfully silenced, a set of empirical guidelines has been proposed for the design of siRNAs (Caplen *et al.*, 2001; Elbashir *et al.*, 2001a; Elbashir *et al.*, 2001b; 2001c). Although these guidelines have allowed for the identification of siRNAs that were efficient in silencing gene expression, it has also been reported, however, that in some instances such siRNAs were ineffective (Harborth *et al.*, 2001). The instability of the siRNA *in vivo*, its inability to interact with components of the RNAi machinery, and the inaccessibility of the target mRNA owing to local secondary structural constraints have been proposed as causes for the failure of these siRNAs (Holen *et al.*, 2002; Bohula *et al.*, 2003; Kretschmer-Kazemi Far and Sczakiel, 2003; Brown *et al.*, 2005). In this study, siRNAs directed against the AHSV-9 VP7 mRNA were identified and compared, using different siRNA prediction software programs, and their design was subsequently refined based on previously published design guidelines. Following analysis of the local secondary structure of the targeted regions on the VP7 mRNA, two VP7 gene-specific siRNAs were subsequently selected and designated siVP7-336 and siVP7-441 (Chapter 3). To evaluate the efficacy of the respective siRNAs to mediate AHSV-9 VP7 gene silencing, a screening vector was constructed by fusing the targeted VP7 sequence and the reporter eGFP gene together to produce recombinant vector pCMV-VP7-eGFP, which could express the VP7-eGFP fusion mRNA. Therefore, the efficacy of the respective VP7 gene-specific siRNAs could be screened rapidly by simply quantifying expression of the eGFP reporter by fluorometry. The result obtained from these

studies indicated that whereas siVP7-336 inhibited VP7-eGFP protein expression by 88%, siVP7-441 silenced VP7-eGFP protein expression by 75%. The high level of protein expression inhibition obtained, especially by using siVP7-336, thus validated the siRNA design strategy used in this investigation. In addition, unlike the bacmid/insect cell expression system, the gene silencing assays were quicker and simpler to perform.

Many studies have reported siRNA-mediated inhibition of the replication of a variety of viruses and it is clear that RNAi represents a potentially powerful tool in controlling virus replication (reviewed in Gitlin and Andino, 2003; Haasnoot *et al*., 2003; Tan and Yin, 2004). Since it is thought that siRNAs target mRNAs containing the same sequences and induce their cleavage, the effect of siVP7-336 and siVP7-446 on the abundance of AHSV-9 VP7 mRNAs in virus-infected BHK-21 cells was investigated at 24 h post-infection by SYBR® Green-based real-time RT-PCR (Chapter 4). Consistent with the VP7-eGFP protein data above, siVP7-336 and siVP7-446 reduced VP7 mRNA levels by 93% and 86%, respectively. Thus, the determination of relative VP7 mRNA levels suggested that suppression of VP7 protein expression was due to specific degradation of the VP7 gene mRNA. The effect of the respective siRNAs on AHSV-9 replication was also investigated. Titration of the supernatants collected from siRNA-treated virus-infected cultures at 24 h post-infection indicated that siVP7-336 was a stringent inhibitor of virus propagation and reduced the infectious viral titres to 16% of that of a control infection performed in the presence of a universal negative siRNA. Taken together, the results indicate that RNAi, induced by the VP7 gene-specific siRNA, efficiently silenced expression of the AHSV VP7 protein by sequence-specific degradation of the VP7 mRNA, thereby resulting in a reduction of infectious viral progeny. The reduction in infectious progeny may be ascribed to aberrant virus particle formation, since VP7 is not only the major structural protein, but is also an important structural determinant of infectious virus particle assembly, probably at the level of capsid assembly (Stuart *et al*., 1998).

This study above represents the first report that siRNA could be used for inhibition of AHSV-9 in cell culture and thus also provides evidence that siRNA has the potential to be developed into a novel antiviral approach, provided that efficient means for *in vivo* siRNA delivery can be developed. However, two aspects in this regard deserve special attention. As discussed previously (Chapter 4), AHSV has a segmented dsRNA genome, which is replicated in subviral particles in the cytoplasm of infected cells and therefore masked at all times during the virus replication cycle. Consequently, the lack of free pools of dsRNA prevents the virus genome to be detected and targeted by the general defense mechanisms of the cell. In addition, the viral positive-strand mRNAs, which are packaged into virions prior to their replication, may be inaccessible to the RNAi

machinery due to their association with RNA-binding proteins and/or virus inclusion bodies in which virus replication and assembly is proposed to occur (Silvestri *et al.*, 2004). Consequently, it may not be possible to block the replication of AHSV genome. Whilst the "resistance" of the viral genomic RNA to siRNA has important implications for antiviral approaches, the introduction of siRNAs preventing replication of the virus (*e.g.* silencing genes involved in the replication of the genome) may possibly aid in shortening the duration of the illness. Furthermore, it has been reported that siRNAs that differ from their target sequence in one or more bases, depending on the position of the mismatch, do not efficiently silence the expression of that gene (Elbashir *et al.*, 2001b). This may be regarded as a serious disadvantage for the use of siRNAs as potential therapeutic antiviral agents, since viruses could escape the RNAi response, as has been reported for HIV (Das *et al.*, 2004) and poliovirus (Gitlin *et al.*, 2002). One way to circumvent this problem may be to use two or more siRNAs targeting different, highly conserved regions on the viral gene or genome (Ji *et al.*, 2003; Liu *et al.*, 2005). In this investigation, results were obtained that indicated that by using a combination of siVP7-336 and siVP7-441, silencing of AHSV-9 VP7 gene expression was not enhanced. Note should, however, be taken that the targets along the VP7 gene were searched for empirically. By adopting a more rational design of the siRNA cocktail (Mittal, 2004), it may be possible to identify efficacious combinations of different siRNAs whereby gene silencing can be enhanced. Nevertheless, an interesting future application of this phenomenon could be to use siRNAs directed to a specific region of the viral gene in order to select for escape mutants that will contain nucleotide changes in the specific targeted region. The siRNAs could thus be used as region-specific mutagens to generate pre-designed mutants for subsequent structure-function analysis.

In conclusion, siRNA-mediated gene-silencing assays have been developed for the specific inhibition of transgene expression in both *S. frugiperda* insect cells and in mammalian BHK-21 cells. Having established technologies whereby the expression of individual AHSV genes can be silenced, future studies should be directed at using these developed assays to investigate not only VP7 protein function, but also the function of other AHSV proteins during the replication cycle of the virus. The assays developed during the course of this investigation could either be applied directly to studying the role of the different proteins or, alternatively, stable cell lines in which all cells in the culture would harbour a silencing siRNA can be constructed. Since one of the limiting steps in using RNAi to silence viral genes is the siRNA transfection efficiency, such an approach may address this problem and could also provide for a longer-lasting gene silencing effect, thus enabling studies to be performed over a longer period.

PUBLICATIONS AND CONGRESS CONTRIBUTIONS DURING THE COURSE OF THIS STUDY

Conference contributions

1. **Burger, L.**, Huismans, H. and Theron, J. (2004). Using dsRNA to inhibit expression of African horse sickness virus VP7 in cell culture. Joint Congress of the South African Microbiology Society and South African Genetics Society, 4-7 April 2004, Stellenbosch, South Africa.

2. **Burger, L.**, Huismans, H., Hall, A.N. and Theron, J. (2004). Construction and structural evaluation of a chimeric VP7 protein of African horse sickness virus. 43rd Annual Conference of the Microscopy Society of Southern Africa, 30 November-3 December 2004, Pretoria, South Africa.

Refereed published conference proceeding

1. **Burger, L.**, Huismans, H., Hall, A.N. and Theron, J. (2004). Construction and structural evaluation of a chimeric VP7 protein of African horse sickness virus. Proceedings of the Electron Microscopy Society of Southern Africa, 34, 43.

Other non-refereed publications

Liesel Burger, Henk Huismans and Jacques Theron. Establishment of an RNA interference (RNAi) assay to inhibit expression of African horse sickness virus VP7 in mammalian cell cultures. Southern Cross Biotechnology newsletter. September 2005.

Manuscript in preparation

Burger, L., Huismans, H. and Theron, J. Silencing African horsesickness virus VP7 protein expression *in vitro* by RNA interference. Submitted to Journal of General Virology.

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APPENDICES

Appendix 1:

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ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGAC      60
 M  V  S  K  G  E  E  L  F  T  G  V  V  P  I  L  V  E  L  D       20

GGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTAC     120
 G  D  V  N  G  H  K  F  S  V  S  G  E  G  E  G  D  A  T  Y       40

GGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACC     180
 G  K  L  T  L  K  F  I  C  T  T  G  K  L  P  V  P  W  P  T       60

CTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAG     240
 L  V  T  T  L  T  Y  G  V  Q  C  F  S  R  Y  P  D  H  M  K       80

CAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTC     300
 Q  H  D  F  F  K  S  A  M  P  E  G  Y  V  Q  E  R  T  I  F      100

TTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTG     360
 F  K  D  D  G  N  Y  K  T  R  A  E  V  K  F  E  G  D  T  L      120

GTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCAC     420
 V  N  R  I  E  L  K  G  I  D  F  K  E  D  G  N  I  L  G  H      140

AAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAAC     480
 K  L  E  Y  N  Y  N  S  H  N  V  Y  I  M  A  D  K  Q  K  N      160

GGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCC     540
 G  I  K  V  N  F  K  I  R  H  N  I  E  D  G  S  V  Q  L  A      180

GACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCAC     600
 D  H  Y  Q  Q  N  T  P  I  G  D  G  P  V  L  L  P  D  N  H      200

TACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTC     660
 Y  L  S  T  Q  S  A  L  S  K  D  P  N  E  K  R  D  H  M  V      220

CTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAA     720
 L  L  E  F  V  T  A  A  G  I  T  L  G  M  D  E  L  Y  K  *      239
```

The complete nucleotide sequence and deduced amino acid sequence of the cloned enhanced green fluorescent protein (eGFP) gene. The translation initiation and termination codons are indicated in bold, while the amino acids are indicated in one-letter code. The ORF begins at position 1 to 3 (ATG) and is terminated at position 718 to 720 (TAA).