Silencing of African horse sickness virus NS2 gene expression using vector-derived short hairpin RNAs

by

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I declare that the thesis/ dissertation, which I hereby submit for the degree MSc. Microbiology at the University of Pretoria, it is my own work and had not previously been submitted by me for a degree at this or any other tertiary institution.

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Date: _____

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SUMMARY

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African horse sickness virus (AHSV), a member of the *Orbivirus* genus within the *Reoviridae* family, has a 10-segment double-stranded (ds)RNA genome enclosed within a double capsid. In addition to seven structural proteins (VP1-VP7), four non-structural proteins (NS1, NS2 and NS3/NS3A) are synthesized in infected cells and are involved in virus morphogenesis. Due to the lack of a reverse genetic system for orbiviruses, analyses regarding AHSV gene function have been limited to the characterization of individual virus proteins following their expression in heterologous expression systems. The phenomenon of RNA interference (RNAi), has, however, revolutionized approaches to study the function of individual genes. RNAi is an evolutionary conserved mechanism by which RNA duplexes, known as short interfering RNA (siRNA), can reduce gene expression through enzymatic cleavage of complementary mRNA. In addition to synthetic siRNA, RNAi can also be induced in mammalian cells by plasmid and viral vector systems that express short hairpin RNAs (shRNAs) that are subsequently processed to siRNAs by the cellular machinery. Consequently, the aim of this investigation was to establish a plasmid DNA vector-based RNAi assay whereby the expression of the AHSV-9 NS2 gene could be suppressed in BHK-21 cells.

Complementary oligonucleotides corresponding to selected AHSV-9 NS2 gene sequences were chemically synthesized, annealed and cloned into the pSUPER shRNA delivery vector,

downstream of the RNA polymerase III H1 promoter. The vector-expressed shRNAs targeted regions within the NS2 gene corresponding to nucleotides 211 to 230 (shRNA-211), 377 to 396 (shRNA-377) and 958 to 977 (shRNA-958), respectively. To determine whether the NS2-directed shRNAs was capable of silencing NS2 protein expression, BHK-21 cells were co-transfected with the respective pSUPER shRNA delivery vectors and a NS2 reporter plasmid, pCMV-NS2-eGFP. Fluorescence microscopy indicated that NS2-eGFP expression was makerdly reduced in these cells compared to cells transfected with the reporter plasmid only, and fluorometry analysis indicated that the level of inhibition mediated by the shRNAs were in excess of 80%. The potential of the NS2-directed shRNAs to reduce the level of NS2 transcripts in AHSV-9 infected BHK-21 cells was also investigated by transfection of the BHK-21 cells with the respective pSUPER shRNA delivery vectors, followed by virus infection. Results obtained by means of semi-quantitative real-time reverse transcriptase-polymerase chain reactions indicated that shRNA-377 interfered the most efficiently with NS2 mRNA expression, and the greatest silencing effect was observed at 24 h post-infection. During the course of this investigation it was also attempted to establish a BHK-21 cell line that stably expressed the NS2-directed shRNA-377. For this purpose, a recombinant pSUPER.Retro.Puro retroviral vector was constructed and following transfection of BHK-21 cells, stable transfectants were selected by growth in the presence of puromycin. Results indicated that although the derived cell line suppressed AHSV-9 NS2 mRNA expression, the plasmid DNA was maintained extrachromosomally. Overall, the results of this investigation have provided evidence that AHSV-9 NS2 gene expression can be suppressed in mammalian cells by vector-derived shRNAs.

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LIST OF ABBREVIATIONS

AHS	African horse sickness
AHSV	African horse sickness virus
Amp	ampicillin
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
CPE	cytopathic effect
CsCl	cesium chloride
Da	Dalton
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
et al.	*et alia* (and others)
FBS	foetal bovine serum
FCS	foetal calf serum
Fig.	figure
GFP	green fluorescent protein
Gm	gentamycin
h	hour
i.e.	*id est* (that is)
IPTG	isopropyl-β-D-thiogalactopyranoside
kan	kanamycin
kb	kilobase pairs

kDa	kilodalton
L	litre
LB	Luria-Bertani
M	molar
mA	milliampere
MEM	Minimum Essential Medium
mg	milligram
min	minute
ml	millilitre
mM	millimolar
MOI	multiplicity of infection
M_r	molecular weight
mRNA	messenger ribonucleic acid
N	normal
ng	nanogram
nm	nanometer
nt	nucleotides
OD	optical density
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
REST	Relative Expression Software Tool
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
RNase	ribonuclease
rpm	revolutions per minute
RT	reverse transcription
RT-PCR	reverse transcription-PCR
s	second
S.D.	standard deviation
SDS	sodium dodecyl sulphate

shRNA	small hairpin RNA
siRNA	small interfering RNA
ss	single stranded
TEMED	N',N',N',N'-tetramethylethylenediamine
U	units
UHQ	ultra high quality
UV	ultraviolet
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
°C	degrees Celsius
µg	microgram
µl	microlitre
µm	micrometre
µM	micromolar
2',5'-AS	2',5'-oligoadenylate synthetase

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CHAPTER ONE

LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

African horse sickness (AHS), of which African horse sickness virus (AHSV) is the aetiological agent, is an infectious, non-contagious, arthropod-borne viral disease of equids. The disease is characterized by clinical signs that develop as a consequence of damage to the circulatory and respiratory systems, thus giving rise to serous effusion and haemorrhage in various organs and tissues (Coetzer and Erasmus, 1994; Mellor and Hamblin, 2004). The first reference to the disease concerns an epidemic that occurred in 1327 in Yemen (Mellor and Hamblin, 2004). However, the virus almost certainly originated in Africa and was first observed on the African continent after the introduction of horses from India in the 16th century for purposes of exploring central and east Africa. The mortality of the disease was close to 90%. The first outbreak of AHS in southern Africa was only recorded in 1719 when over 1 700 animals succumb to the disease in the then Cape of Good Hope. Subsequently, over the next 217 years, at least 10 major outbreaks of AHS have been recorded in southern Africa. The most severe outbreak occurred in the Cape of Good Hope in 1854-1855 in which more than 70 000 horses died (Coetzer and Erasmus, 1994). The frequency and severity of the outbreaks have, however, declined over the last century, probably due to improved surveillance and strict zoning measures as well as vaccination of horses with polyvalent, live attenuated vaccines (Bosman *et al.*, 1995; Mellor and Hamblin, 2004). Nevertheless, based on its potential economic and international importance, AHS has been classified as a list A disease by the Office International des Epizootics (OIE) (Coetzer and Erasmus, 1994; Mellor and Hamblin, 2004).

Much of the pioneering research on AHS has been performed by Sir Arnold Theiler during the early 20th century. In 1900, he demonstrated the filterability of the pathogen through Berkefield and Chamberland filters, thereby demonstrating that the pathogen was indeed a virus. Theiler's research also indicated that there existed immunologically distinct strains of the AHS virus agent, since immunity acquired against one strain did not always protect the horse when challenged by a heterologous virus strain. In 1903, Theiler and Pitchford-Watkins established that AHSV may be transmitted by biting insects and, in 1921, Theiler reported the first detailed descriptions of the clinical signs and lesions produced by infections with AHSV (reviewed in Coetzer and Erasmus, 1994). During the late 1960s and 1970s, several studies were undertaken aimed at characterizing the structure and morphology of AHSV (Verwoerd and Huismans, 1969; Oellerman *et al.*, 1970; Bremer, 1976). Based on the results of this

research, it was found that AHSV could be classified as a member of the genus *Orbivirus* in the family *Reoviridae*. This family is comprised of a large number of viruses with segmented double-stranded (ds)RNA genomes and the virus particles have an inner protein capsid, and one (or two) distinctive outer capsids (Urbano and Urbano, 1994).

With the advent of genetic engineering technologies and the development of diverse gene expression systems, much progress has been made regarding structure-function relationships of different AHSV genes and encoded gene products (Vreede and Huismans, 1994; Uitenweerde *et al.*, 1995; Maree and Huismans, 1997; Van Staden *et al.*, 1998; de Waal and Huismans, 2005). Recently, RNA interference (RNAi) has generated great excitement in the molecular biology research community. RNAi is a post-transcriptional mechanism of sequence-specific gene silencing that is initiated by dsRNA (Fire *et al.*, 1998; Sharp, 1999; Hammond *et al.*, 2001a). The target mRNA can be degraded through the introduction of either 21-nucleotide synthetic duplexes of RNA (siRNA) or by transcription of DNA precursors into short hairpin RNAs (shRNAs) that are homologous to a target sequence (Elbashir *et al.*, 2002; Wadhwa *et al.*, 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; Campagna *et al.*, 2005).

In this review of the literature, aspects relating to AHSV epidemiology, pathogenecity, structure and replication will be discussed. This will be followed by a discussion of RNAi and its application in molecular virology, and a brief description of the aims of this investigation.

1.2 TAXONOMIC CLASSIFICATION OF AHSV

African horse sickness virus (AHSV) is a member of the *Orbivirus* genus, one of nine genera within the family *Reoviridae*, which encompasses vertebrate, arthropod and plant pathogens (Gorman, 1992; Urbano and Urbano, 1994). The name "Orbivirus" was proposed by Borden *et al.* (1971) to describe a number of arthropod-borne viruses that based on morphological and physicochemical properties formed a distinct group in the *Reoviridae*. Since their negatively-stained virus particles had large doughnut-shaped capsomers, the genus was named *Orbivirus* (from the latin "orbis" meaning ring or circle). To date, there are 19 recognized serogroups of orbiviruses, as based on biochemical and immunological tests (Calisher and Mertens, 1998).

Viruses within each serogroup can be classified into serotypes on the basis of antibody-mediated neutralization of viral infectivity. There are presently nine recognized serotypes of AHSV (McIntosh, 1958; Howell, 1962).

Members of the family *Reoviridae* have several basic features in common. In general, the viruses are icosahedral, non-enveloped particles of *ca.* 60-80 nm in diameter with a double-layered capsid containing a genome of 10-12 segments of double-stranded (ds)RNA (Verwoerd *et al.*, 1972; Verwoerd *et al.*, 1979; Urbano and Urbano, 1994). All the viruses essentially replicate in the cytoplasm of host cells without complete uncoating of viral particles and produce 5' end-capped, non-polyadenylated mRNAs (Urbano and Urbano, 1994; Roy, 2001), and form cytoplasmic inclusion bodies (Lecatsas, 1968; Murphy *et al.*, 1971). Furthermore, members of the *Reoviridae* are relatively heat stable, resistant to lipid solvents and, unlike orbiviruses that lose infectivity at low pH, are resistant over a wide pH range (Murphy *et al.*, 1971; Verwoerd *et al.*, 1979).

1.3 EPIDEMIOLOGY, TRANSMISSION AND PATHOGENESIS OF AHSV

African horse sickness (AHS) is an infectious but non-contagious viral disease of equids (horses, donkeys and mules), with horses being the most susceptible (Tomori *et al.*, 1992). Although zebra rarely exhibit clinical signs of AHSV infections, they have long been considered the natural vertebrate host and reservoir of AHSV, and are believed to play an important role in the persistence of the virus in Africa (Erasmus *et al.*, 1978; Barnard, 1998). AHSV is endemic in tropical and sub-tropical areas of Africa south of the Sahara, occupying a broad band stretching from Senegal in the west to Ethiopia and Somalia in the east, and extending as far south as northern South Africa (Mellor and Hamblin, 2004). Although AHS was believed to be confined largely to sub-Saharan Africa, except for occasional excursions into North Africa, there have been several outbreaks of the disease in countries of the Persian Gulf and Middle East (Howell, 1960; Brown and Dardiri, 1990; Mellor, 1993). Outbreaks of AHS have also occurred in Spain and Portugal (Lubroth, 1988). Of the nine AHSV serotypes, serotypes 1 to 8 are found only in restricted areas of sub-Saharan Africa, while serotype 9 is more widespread and has been responsible for almost all epidemics outside Africa, the exception being the Spanish and Portuguese outbreaks that were due to serotype 4 (Coetzer and Erasmus, 1994).

AHSV had long been thought to be transmitted between its equid hosts by biting arthropods and studies have shown that biting haematophagous midges of the *Culicoides* genus are the most important vectors of AHSV (Du Toit, 1944; Wetzel *et al.*, 1970; Mellor *et al.*, 1975). An adult *Culicoides* becomes infective eight days after feeding on a viraemic animal and stays infected until death. The major vector of AHSV is *C. imicola*, which occurs throughout Africa, much of Southeast Asia and southern Europe (Mellor, 1994). More recently, *C. bolitinos* has also been identified as a potential vector of AHSV (Venter *et al.*, 2000). *C. bolitinos* has a wide distribution in southern Africa and is common in cooler highland areas where *C. imicola* is rare.

After transfer of AHSV by the bite of infective midges, AHSV is transported to the lymph nodes of the animal where initial virus replication takes place. The viruses are subsequently disseminated throughout the body via the blood (primary viraemia) and virus replication in target organs and endothelial cells give rise to a secondary viraemia (Mellor and Hamblin, 2004). According to the extent and severity of clinical symptoms caused by the infection, the disease is classified into four distinct forms. These are the pulmonary or peracute form, the cardio-pulmonary or mixed form, the cardiac or subacute form and horse sickness fever (Coetzer and Erasmus, 1994). In the pulmonary form, a fever is first detected, followed by coughing, dyspnoea and the appearance of a discharge of fluid from the nostrils. Death usually occurs several hours after the onset of dyspnoea and less than 5% of horses with this form of disease recover. The mixed pulmonary and cardiac form of the disease is the most common form of the disease with horses sometimes exhibiting signs of respiratory distress. The mortality rate is *ca.* 70% with death occurring within three to six days after onset of fever. With the cardiac form, the horse suffers subcutaneous swelling of the head, neck and the supraorbital fossae. Approximately 35% of affected horses recover, but mortality rates exceeding 50% have been reported. The horse sickness fever form of the disease is characterized by horses displaying mild to moderate fever, scleral infection and mild depression, which is followed by complete recovery (Coetzer and Erasmus, 1994).

Although there is no efficient treatment for AHS, horses in southern Africa are vaccinated annually with polyvalent, live attenuated vaccines in an attempt to prevent disease and control outbreaks (Coetzer and Erasmus, 1994; Sanchez-Viscaino, 2004). Alternative vaccines are currently being researched and 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 VP2 and VP5 (Martinez-Torrecuadrada *et al.*, 1996; Roy *et al.*, 1996; Stone-Marchat *et al.*, 1996; Scanlen *et al.*, 2002). However, the subunit vaccines have not yet been commercialized, which may be indicative of the cost and/or difficulties associated with their large-scale production.

1.4 THE STRUCTURE OF AHSV

Bluetongue virus (BTV), the prototype orbivirus, has been characterized extensively at both the molecular and structural levels. Thus, in the following sections, information on BTV will be supplemented with that obtained from similar studies undertaken on AHSV.

1.4.1 Virion structure

The AHSV virion is non-enveloped with two concentric protein layers, enclosing a dsRNA genome consisting of 10 segments (Oellerman *et al.*, 1970; Roy *et al.*, 1994b). The inner shell is termed the core and is composed of two major proteins, *i.e.* VP3 and VP7. The core encloses three minor proteins, *i.e.* VP1, VP4 and VP6, and the RNA genome. The outer shell (outer capsid) consists of two other major proteins, *i.e.* VP2 and VP5 (Roy *et al.*, 1994b).

The structure of the AHSV particle is comparable to that of BTV of which the structure of single- and double-shelled virus particles has been determined by cryoelectron microscopy and by X-ray crystallography (Hewat *et al.*, 1992a; Prasad *et al.*, 1992; Grimes *et al.*, 1998). The core particle can be segregated into two distinct layers. A thin inner layer is formed from 120 large triangular plates of VP3 to from a complete shell, while minimizing conformational distortion. The internal scaffold of VP3 is stabilized by the outer layers of the core that comprise a coat of 260 VP7 trimers. The core contains the dsRNA genome and the transcription complexes consisting of VP1 (12 copies), VP4 (20 copies) and VP6 (60 copies), each of which plays a significant role in genome RNA replication (Stuart and Grimes, 2006). Cryoelectron microscopy and analyses have also revealed that the outer shell of BTV is highly ordered (Hewat *et al.*, 1992a), consisting of 120 globular VP5 regions that are located upon each of the six-membered rings of the VP7 trimers. Moreover, “sail”-shaped spikes of VP2 form triskelion-type motifs that cover nearly all of the VP7 trimers. Together, VP2 and VP5 form a continuous layer surrounding the core, except for holes on the 5-fold axis (Stuart *et al.*, 1998). A schematic diagram of the BTV particle is presented in Fig. 1.1.

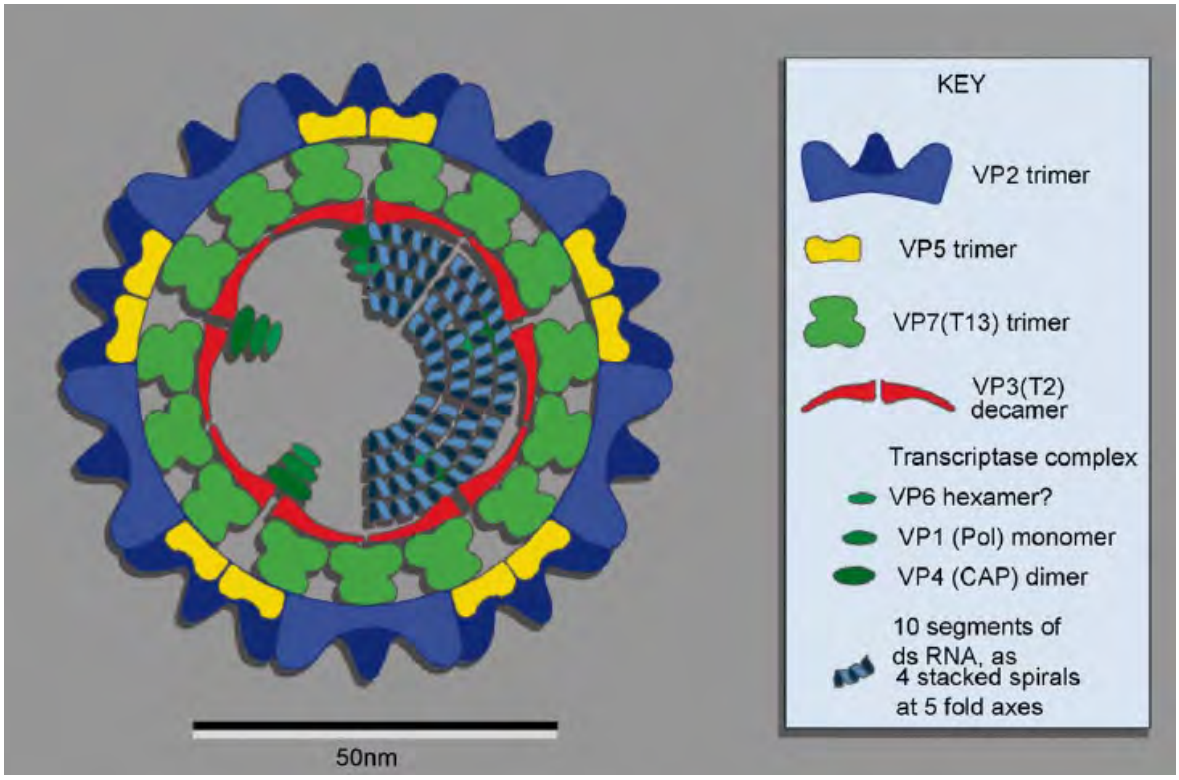

Fig. 1.1 Schematic diagram of the BTV particle (Mertens, 2004). The core particle, which comprises VP3 and VP7, encloses the three minor core proteins, namely VP1, VP4 and VP6, and the 10 dsRNA viral genome segments. The core is surrounded by the outer capsid composed of VP2 and VP5.

1.4.2 Viral genome

The genome of AHSV consists of 10 dsRNA segments (designated L1 to L3, M4 to M6 and S7 to S10) that range in size from 2.53 to 0.2 × 10^6 Da, with a total molecular weight of *ca.* 13 × 10^6 Da (Bremer, 1976; Gorman *et al.*, 1977; Bremer *et al.*, 1990). These segments can be resolved by agarose or polyacrylamide gel electrophoresis and are numbered in the order of migration. Each genome segment is monocistronic, except for S10, which encodes the two related non-structural proteins NS3 and NS3A (Van Staden and Huismans, 1991; Grubman and Lewis, 1992). The viral genome segments contain conserved 5' (5'-GUUAAA……) and 3' (……ACUUAC-3') terminal hexanucleotides, a feature that is typical for viruses in the *Reoviridae* family. In addition, each genome segment has unique complementary inverted repeat sequences (Rao *et al.*, 1983; Roy, 1992; Roy *et al.*, 1994b), which may be important for initiation of transcription and/or the sorting and assembly of genome segments during replication (Cowley *et al.*, 1992; Pritlove *et al.*, 1995).

1.4.3 Viral proteins

The 10 AHSV dsRNA genome segments with their encoded proteins and possible functions are summarized in Table 1.1.

1.4.3.1 Outer capsid proteins

The icosahedral core of the virion is surrounded by an outer capsid composed of VP2 and VP5, and in the case of BTV makes up *ca*. 40% of the total virion mass (Huismans and Van Dijk, 1990). VP2, the outer most of the proteins, is the serotype-specific antigen (Huismans and Erasmus, 1981; Kahlon *et al.*, 1983) and viral haemagglutinin (Cowley and Gorman, 1987; French *et al.*, 1990). Moreover, VP2 is involved in attachment of the virus to cells (Hassan and Roy, 1999) and has been reported to bind to sialic acid moieties of cellular receptors prior to internalization of the virus particle (Hassan and Roy, 1999). Injection of animals with purified BTV VP2 (Huismans *et al.*, 1987a) or baculovirus-expressed VP2 (Inumaru and Roy, 1987) elicits neutralizing antibodies that confer protection in sheep against homologous virus challenge. Neutralizing epitopes have been mapped on AHSV VP2 (Bentley *et al.*, 2000; Martinez-Torrecuadrada *et al.*, 2001) and antibodies raised in rabbits to AHSV-4 have been reported to neutralize a virulent strain of AHSV-4 (Martinez-Torrecuadrada *et al.*, 1994).

Table 1.1 AHSV genome segments and encoded proteins

Segment (no. of bp)	Proteins	Protein size (M_r in Da)	Copy number/ particle	Location	Function
L1 (3 954)	VP1	1 302 (149 588)	~ 12	Within the subcore	RNA-dependant RNA polymerase
L2 (2 926)	VP2	956 (111 112)	180	Outer capsid	Serotype-specific antigen, neutralization, cell attachment, involved in determination of virulence
L3 (2 770)	VP3	901 (103 304)	120	Inner capsid	Interacts with internal minor proteins, controls overall size and organization of capsid structure
M4 (1 981)	VP4	644 (76 433)	20	Within the subcore	Capping enzyme, guanyltransferase, methyltransferase
M5 (1 769)	NS1	552 (64 445)	0	Non-structural protein, cytoplasm forms tubules	Unknown
M6 (1 638)	VP5	526 (59 163)	360	Outer capsid	Helps determine virus serotype, can mediate cell fusion and has a role in cell entry
S7 (1 156)	VP7	349 (38 548)	780	Inner capsid	Involved in cell entry and core particle infectivity
S8 (1 124)	NS2	357 (40 999)	0	Non-structural protein, cytoplasm forms virus inclusion bodies	Viral inclusion body matrix protein, binds ssRNA, NTPase activity
S9 (1 046)	VP6	328 (35 750)	60	Inside sub-core	Binds ssRNA and dsRNA, helicase, NTPase
S10 (822)	NS3 NS3A	229 (25 572) 216 (24 020)	0 0	Non-structural protein, viral protein in cell membrane	Membrane proteins involved in viral cell exit, may be involved in determination of virulence

In contrast to VP2, very little is known about the function of VP5. Despite VP5 being the group-specific antigen and being located in the outer capsid, it does not demonstrate any neutralization activity on BTV replication *in vitro* (Marshall and Roy, 1990). However, when VP5 is used in conjunction with VP2 in vaccination studies, the combination of proteins has been reported to enhance protective immunity (Roy *et al.*, 1994a; Martinez-Torrecuadrada *et al.*, 1996). It was suggested that VP5 may modulate the conformation of VP2 and thus influence its protective efficiency. In contrast to BTV, AHSV VP5 is able to induce neutralizing antibodies, albeit at lower levels than VP2 (Martinez-Torrecuadrada *et al.*, 1999).

1.4.3.2 Major core proteins

The major core proteins, VP3 and VP7, form the outer layer of the viral core particle. The VP3 protein forms an inner scaffold for the deposition of VP7 on the core (Stuart *et al.*, 1998), as has been evidenced by assembly of VP3 and VP7 of BTV (French *et al.*, 1990; Kar *et al.*, 2004) and AHSV (Maree *et al.*, 1998) into cores by co-expression using recombinant baculoviruses. The structure and assembly pathway of the BTV VP3 subcore has been reported (Grimes *et al.*, 1998; Kar *et al.*, 2004). VP3 was shown to have three distinct domains, *i.e.* an apical, a carapace and a dimerization domain. The latter domain mediates the formation of VP3 dimers, which pack together in decamers to form the icosahedral structure of the VP3 layer. Moreover, BTV VP3 also binds to RNA and therefore suggests that VP3 may play a role during the early stages of virion assembly (Loudon and Roy, 1992).

The VP7 protein is the most abundant protein in the core particle (Huismans and Van Dijk, 1990) and self-assembles into trimers (Basak *et al.*, 1992), which form the outer shell of the core. The crystal structure of BTV VP7 has been solved (Grimes *et al.*, 1995) and revealed that VP7 comprises an upper β-barrel and lower α-helical domain, separated by a hinge region. The hinge region allows for conformational twisting to enable interaction between VP7 monomers to form the trimeric structures of the BTV core (Basak *et al.*, 1992). The crystal structure of the β-barrel (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 both viral VP7 proteins retain a surface-exposed RGD motif, which in the case of BTV, has been shown to be responsible for attachment of core particles to *Culicoides* cells (Tan *et al.*, 2001). In contrast to BTV, the AHSV VP7 protein forms flat hexagonal structures in the cytoplasm of both virus-infected (Burroughs *et al.*, 1994) and recombinant baculovirus-infected cells (Chuma *et*

al., 1992; Maree and Paweska, 2005). Although the functional significance of the VP7 crystalline structures is not yet known, it is thought to represent a by-product rather than an essential component of the AHSV replication cycle (Burroughs *et al.*, 1994).

1.4.3.3 Minor core proteins

The three minor core proteins, VP1, VP4 and VP6, are candidates for the virus-directed RNA polymerase and associated proteins, which are responsible for transcription of the 10 viral mRNAs that are transcribed during the infectious cycle. The amino acid sequence of the largest viral protein, VP1, not only contains a GDD motif, which is characteristic of RNA polymerases, but also displays homology with other known RNA polymerases, including the β-subunit of *Escherichia coli* RNA polymerase (Roy *et al.*, 1988; Vreede and Huismans, 1998). Moreover, by making use of lysates prepared from cells infected with a recombinant baculovirus expressing the BTV VP1 protein, it was demonstrated that poly(A) synthesis can occur when the extract is provided with a poly(A) primer and poly(U) template (Urakawa *et al.*, 1989). It has also been demonstrated that purified BTV VP1 protein is capable of synthesizing minus-strand RNA from a BTV-derived positive-stranded RNA template (Ramadevi *et al.*, 1998). Based on its location and molar ratio in the core (12 molecules per virion), as well as bioinformatic and functional analyses, VP1 is thus the prime candidate for the virion RNA-dependant RNA polymerase. The 5' ends of the viral mRNA species are believed to be capped and methylated during transcription, thus resulting in stabilization of the viral mRNA synthesized during infection (Roy, 1992). The VP4 protein of BTV has been reported to demonstrate guanylyl transferase (Le Blois *et al.*, 1992) and methyltransferase (Ramadevi *et al.*, 1998) activities. Furthermore, VP4 also binds to GTP and displays nucleoside triphosphate phosphohydrolase (NTPase) activity, which is considered to be of importance for transcription and dsRNA processing (Ramadevi and Roy, 1998). The VP6 minor core protein of BTV (Roy *et al.*, 1990) and AHSV (de Waal and Huismans, 2005) is capable of binding both viral and non-viral ssRNA, as well as dsDNA. The nucleic acid-binding activity is mediated by two nucleic acid-binding domains that mapped to a region in the centre of the protein and to the carboxy-terminus (Hayama and Li, 1994; de Waal and Huismans, 2005). BTV VP6 has also been shown to be an ATP-dependant RNA helicase, which catalyses the unwinding of duplex RNA in the presence of divalent cations (Staeuber *et al.*, 1997). Thus, VP6 may be involved in unwinding the dsRNA genome prior to the initiation of transcription or may be involved in the encapsidation of the RNA.

1.4.3.4 Non-structural proteins

Four non-structural proteins are synthesized in the virus-infected cells. The two major non-structural viral proteins NS1 and NS2 are synthesized abundantly (Huismans and Els, 1979), whereas the third non-structural protein NS3 and the co-linear, truncated NS3A protein are synthesized in low amounts during virus replication (Van Dijk and Huismans, 1988; French *et al.*, 1989; Van Staden and Huismans, 1991). The synthesis of NS1 and NS2 in virus-infected cells coincides with the appearance of two virus-specific structures, *i.e.* tubules and granular viral inclusion bodies (VIBs) (Lecatsas, 1968).

Tubules are composed entirely of the NS1 protein (Huismans and Els, 1979; Urakawa and Roy, 1988; Maree and Huismans, 1997). The NS1 amino acid sequence, structure and biophysical character of the tubules differ between BTV and AHSV (Huismans and Els, 1979). The BTV tubules have a diameter of 52.3 nm, are *ca.* 1 000 nm in length and they are formed by a helically coiled ribbon of NS1 dimers (Hewatt *et al.*, 1992b). In contrast, AHSV tubules have a diameter of 23 nm and vary in length up to 4 µm with a cross-weave appearance (Maree and Huismans, 1997). No function has yet been assigned to NS1, but it has been proposed that the protein may be a major determinant of BTV pathogenesis in the vertebrate host since it augments virus-cell association that ultimately leads to lysis of the infected cell (Owens *et al.*, 2004). It is also believed that the tubules may serve as a biologically inert storage form of NS1 that had been utilized in a prior stage of viral morphogenesis (Huismans and Els, 1979).

The NS2 protein is the predominant component of VIBs (Hyatt and Eaton, 1988; Brookes *et al.*, 1993) and expression of NS2, in the absence of other viral proteins in both insect and mammalian cells, results in the formation of inclusion bodies (IBs) that are indistinguishable of VIBs found in virus-infected cells (Thomas *et al.*, 1990; Uitenweerde *et al.*, 1995; Modrof *et al.*, 2005). NS2 is also the only virus-specific protein that is phosphorylated in infected cells (Huismans *et al.*, 1987b; Devaney *et al.*, 1988). Although the significance of NS2 phosphorylation is not understood completely, a recent report has indicated that BTV NS2 is phosphorylated by casein kinase II (CKII) and that phosphorylation of NS2 is important for VIB formation (Modrof *et al.*, 2005). In addition to the aforementioned properties, NS2 has a strong affinity for ssRNA but not for dsRNA, suggesting that it may have a role in the recruitment and packaging of viral ssRNA prior to encapsidation (Huismans *et al.*, 1987b).

Moreover, BTV RNAs are preferentially bound over non-specific RNAs suggesting that NS2 may have a role in the recruitment of viral mRNA for replication (Theron and Nel, 1997; Lymperopoulos *et al.*, 2003; Markotter *et al.*, 2004). Interestingly, BTV NS2 displays phosphohydrolase (NTPase) activity and can bind and hydrolyse ATP and GTP to their corresponding nucleotide monophosphates (Horscroft and Roy, 2000; Taraporewala *et al.*, 2001). It has been suggested that the NTPase action of NS2 may play a role in providing energy for the assortment, movement, packaging or condensation of bound ssRNA (Horscroft and Roy, 2000).

The non-structural proteins NS3 and NS3A are both encoded by the S10 genome segment (French *et al.*, 1989; Van Staden and Huismans, 1991; Van Staden *et al.*, 1995). Biochemical and immunological analyses have indicated that NS3A is synthesized by an alternative down-stream in-frame AUG translation initiation site. Moreover, deletion of the first AUG codon abolishes synthesis of NS3 but not that of NS3A (Wu *et al.*, 1992). Analysis of the encoded amino acid sequence of the S10 genome segment has revealed the presence of two hydrophobic regions that may serve as transmembrane domains (Van Staden and Huismans, 1991; Van Niekerk *et al.*, 2001). The BTV NS3, in contrast to that of AHSV, has been reported to be glycosylated (Wu *et al.,* 1992) and it was suggested that *N*-linked glycosylation of the NS3 protein may serve to protect it from degradation (Bansal *et al.*, 1998). Within BTV-infected cells the NS3 proteins have been observed to co-localize with BTV particles within disrupted regions of the plasma membrane, suggesting that the NS3 protein may be involved in the release of BTV from infected cells (Hyatt *et al.*, 1989). In addition, it has been reported that baculovirus-expressed virus-like particles (VLPs) are only released in the presence of NS3 protein (Hyatt *et al.*, 1992), thus furthermore suggesting a role for NS3 proteins in virus release. Similarly, the AHSV NS3 is an integral membrane protein and is also localized to sites of AHSV release (Stoltz *et al.*, 1996).

1.5 ORBIVIRUS REPLICATION AND MORPHOGENESIS

Unlike other vertebrate-infecting members of the family *Reoviridae*, unravelling of the orbivirus replication cycle at the molecular level is compounded by their ability to replicate in both vertebrate hosts and arthropod vectors. Moreover, the effect of orbivirus replication in these distinct hosts types is markedly different. For BTV, replication of the virus in insect

cells results in persistent infection with little or no cytopathic effect (Homan and Yunker, 1988). However, infection of mammalian cells results in cell death (Huismans, 1979; Wechsler and McHolland, 1988). Although it is likely that the basic replication strategies may be similar in the different host types, orbiviruses must have evolved specific replication mechanisms to enable the survival of its arthropod vector to ensure infection of a new vertebrate host. Using BTV as a model for orbivirus replication and morphogenesis, four major events in the replication cycle of orbiviruses have been identified and are discussed below in greater detail. These events 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 virus to and release from the cell surface (Fig. 1.2) (Gould and Hyatt, 1994; Roy, 2001; Mertens, 2004).

BTV rapidly adsorbs to susceptible mammalian cells, with maximal adsorption occurring within 20 min (Huismans *et al.*, 1983). Although the cellular receptors to which BTV binds have not yet been identified, it has been shown that the binding and internalization of BTV in mammalian cells is mediated by the outer capsid protein VP2 (Hassan and Roy, 1999). In addition, these studies have also indicated that the VP2 of BTV attaches to sialoglycoproteins of mammalian cells prior to internalization. In contrast to mammalian cells, BTV virions bind *Culicoides* cells with low efficiency; however, core particles are highly infectious (Mertens *et al.*, 1996; Tan *et al.*, 2001). The results suggest therefore that the outer core protein VP7 is responsible for binding to insect cell receptors.

Following adsorption, BTV is transported into the cytoplasm of the cell via receptor-mediated endocytosis that involves invagination of the cellular membrane to form a coated vesicle within the cell and these endocytotic vesicles fuse to form endosomes (Hyatt *et al.*, 1989; Eaton *et al.*, 1990). Within the vesicle, the outer capsid proteins are lost, presumably due to the acidic conditions within the endosome, and the transcriptionally active core particles are released into the cytoplasm of infected cells (Van Dijk and Huismans, 1980; Huismans *et al.*, 1987c). It has been suggested that VP5 may be responsible for the release of transcriptionally active cores from the endosomes, since removal of the VP2 from the virions results in the exposure of helical membrane-destabilizing domains of VP5 (Hassan *et al.*, 2001). The replication of BTV is initiated by the synthesis and extrusion of capped and methylated mRNA from transcriptionally active cores within the cytoplasm. The first viral proteins can be detected between 2-4 h after infection, after which the rate of viral protein synthesis

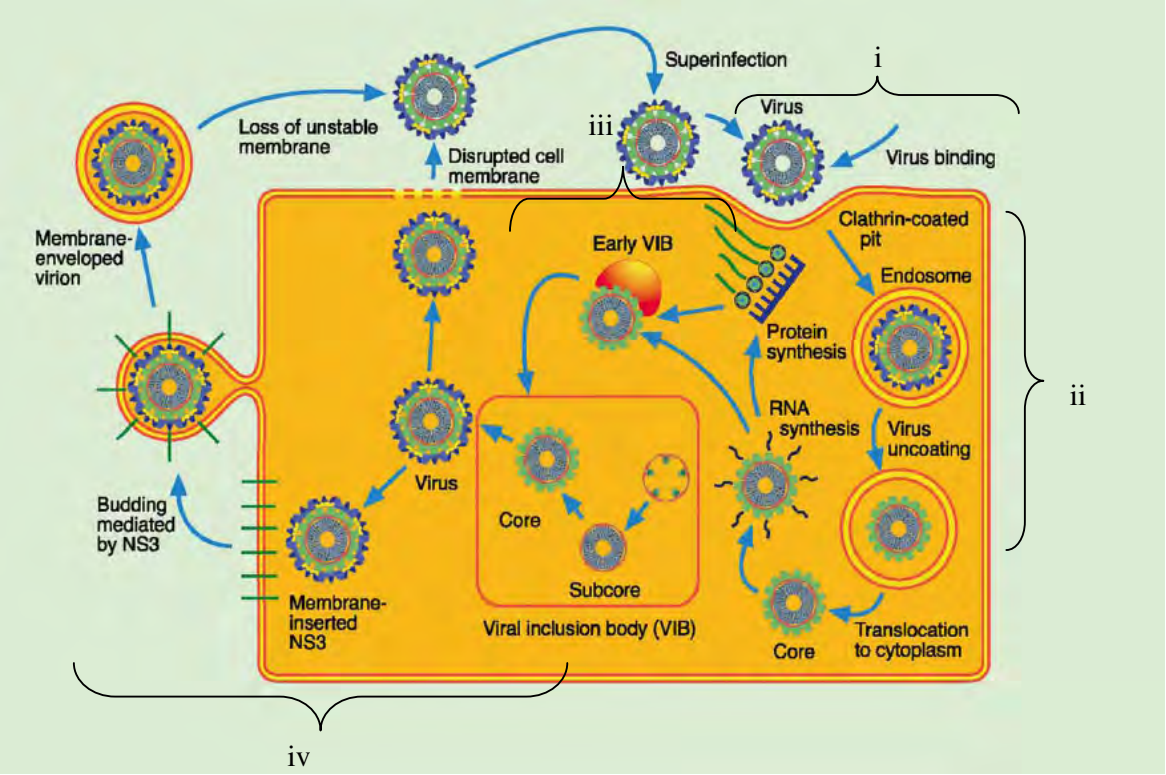

Fig. 1.2 Schematic diagram representing the replication cycle of BTV (Mertens, 2004). The diagram illustrates the four major events of the orbivirus replication, namely (i) adsorption and penetration, (ii) uncoating and formation of replicative complexes, (iii) formation of virus tubules and virus inclusion bodies, and (iv) movement of virus to and release from the cell surface.

increases rapidly until 11-13 h post-infection, and then slows down until cell death (Huismans, 1979; Huismans and Van Dijk, 1990). The infection and replication of BTV in mammalian cells results in the complete shutdown of host cell protein synthesis by 11 h post-infection, although the mechanism by which BTV causes this effect is unknown (Huismans, 1979).

Soon after the initiation of transcription of BTV mRNAs, a granular matrix structure accumulates near the core particles (Hyatt *et al.*, 1987). These matrix structures are known as the virus inclusion bodies (VIBs) and as the infection progresses, these VIBs increase both in size and number (Eaton *et al.*, 1990). Newly synthesized viral transcripts, the four subcore viral proteins (VP1, VP3, VP4 and VP6), as well as assembled cores and subcores have been identified in the VIBs and thus appear to be the sites of orbivirus replication and early viral assembly (Hyatt and Eaton, 1988). More recently, co-expression of the BTV structural proteins with NS2 have indicated that VP7 requires co-expression of VP3 to be recruited to the VIBs and that neither of the outer capsid proteins VP5 and VP2 have an affinity for the VIBs (Modrof *et al.*, 2005; Kar *et al.*, 2007). Therefore, it would appear that progeny core particles are first produced in the VIBs, then moved to periphery of the VIBs where they are coated by the outer capsid proteins VP5 and VP2 (Kar *et al.*, 2007). In addition to VIBs, NS1-rich tubules form part of the 'insoluble' phase of the cell and become a characteristic structure of the cell from an early stage of infection (Huismans and Els, 1979; Eaton *et al.*, 1988). It has recently been reported that NS1, which is produced abundantly in infected cells, is involved in the viral replication cycle through augmentation of the virus-cell association that leads to lysis of infected cells (Owens *et al.*, 2004).

BTV utilizes different strategies to escape from vertebrate and invertebrate cells. Investigations regarding virus release from mammalian cells have demonstrated a strong correlation between the presence of NS3 and NS3A, and virus release (Hyatt *et al.*, 1989; Hyatt *et al.*, 1992). In addition, virions have been demonstrated to leave infected cells in two ways. Early after the infection, progeny virions have been observed to bud through the plasma membrane, acquiring a transient envelope. Alternatively, egress of virions can be accomplished by non-enveloped particles by extrusion through locally disrupted plasma membrane surfaces (Hyatt *et al.*, 1989). Although virus release is observed in mammalian cells, virions remain mainly associated with cellular components and only a minority of particles are found in the extracellular medium (less than 10%), presumably due to release as

a consequence from cytopathic effect (CPE) and death of infected cells (Guirakhoo *et al.*, 1995). In contrast to mammalian cells, infection of insect cells results in persistent infection with no CPE and the majority of progeny virions (90%) are released into the supernatant (Guirakhoo *et al.*, 1995). The increased release of progeny virions in insect cell culture is also associated with increased NS3/NS3A expression (Guirakhoo *et al.*, 1995). This data indicates that NS3 plays an important role in the release of progeny virions from infected cells (Hyatt *et al.*, 1989; Hyatt *et al.*, 1992; Stoltz *et al.*, 1996; Wirblich *et al.*, 2006).

1.6 RNA INTERFERENCE (RNAi)

Typically, targeted inhibition of mammalian gene expression has been achieved primarily by approaches such as homologous recombination, antisense oligonucleotides and ribozymes (Robishaw *et al.*, 2004; Nahreini *et al.*, 2004; Scanlon *et al.*, 2005). However, in the past few years, RNA interference (RNAi) has emerged as the primary means whereby specific genes in mammalian systems can be suppressed or silenced. RNAi, a process by which dsRNA 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 is related to the previously described post-translational gene silencing (PTGS) mechanism of co-suppression in plants (Napoli *et al.*, 1990) 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 (Agrawal *et al.*, 2003). Although a physiological role for RNAi in mammals has not yet been established, it is thought that the natural function of RNAi is to protect the host against invasion by mobile genetic elements such as transposons and viruses, (Tabara *et al.*, 1999), and to maintain normal growth and development (Grishok *et al.*, 2001; Bernstein *et al.*, 2003). Based on its apparent universal applicability, high specificity and simplicity, RNAi has emerged as a powerful tool for analyzing gene function in diverse groups of organisms, including viruses, and it has been proposed that RNAi may provide a new therapeutic approach for controlling viral infections (Haasnoot *et al.*, 2003; Colbère-Garapin *et al.*, 2005, Stram and Kuzntzova, 2006). In this part of the literature review, aspects relating to the mechanism underlying RNAi, its potential as a research tool and its application in the inhibition of viral replication will be addressed.

1.7 MECHANISM OF RNAi

Biochemical and genetic analyses have provided a mechanistic understanding of RNAi-mediated gene silencing (Agrawal *et al.*, 2003; Meister and Tuschl, 2004; Jaronczyk *et al.*, 2005). In the first step, referred to as the RNAi initiating step, long dsRNA is typically cleaved into discrete 21- to 23-nucleotide (nt) RNA fragments that are termed small interfering RNA (siRNA). In the second step, these siRNAs are assembled into a RNA-induced silencing complex (RISC), which degrades the homologous single-stranded mRNA. A two-step mechanistic model for RNAi-mediated gene silencing is indicated in Fig. 1.3.

1.7.1 Processing of dsRNA into siRNA

Long dsRNAs present in the cell cytoplasm are first processed to siRNAs by the RNase III-like enzyme Dicer (Hammond *et al.*, 2000; Bernstein *et al.*, 2001). Dicers are 200-kDa multidomain proteins, which have an amino-terminal DExH/DEAH RNA helicase domain, a PAZ (PIWI-Argo-Zwille/Pinhead) domain, a tandem repeat of RNase III catalytic domain sequences (RIIIa and RIIIb), and a carboxy-terminal dsRNA-binding motif (Provost *et al.*, 2002; Jaronczyk *et al.*, 2005). Based on results obtained from studying the effects of a mutant human Dicer enzyme, Zhang *et al.* (2004a) reported that Dicer has only one dsRNA processing centre, containing two RNA cleavage sites, and produces siRNA duplexes with 2-nt 3' overhangs by cleaving two nearby phosphodiester bonds on opposite RNA strands. It was subsequently proposed that Dicer functions through intramolecular dimerization of its RNA catalytic domain sequences, assisted by the flanking PAZ and RNA-binding domains. 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 melanogaster* (Zamore *et al.*, 2000), the recombinant human Dicer was reported to generate siRNAs from dsRNA efficiently in the presence of Mg^{2+} and the absence of ATP (Zhang *et al.*, 2002). Notably, the length of siRNAs produced in different organisms varies between 21 and 28 nucleotides and may reflect structural differences of the various Dicer orthologs. Furthermore, the distinct size and structure of siRNA has been proposed to reflect the geometric spacing between the active sites of dsRNA-bound dimers of Dicer during dsRNA processing (Zamore, 2001).

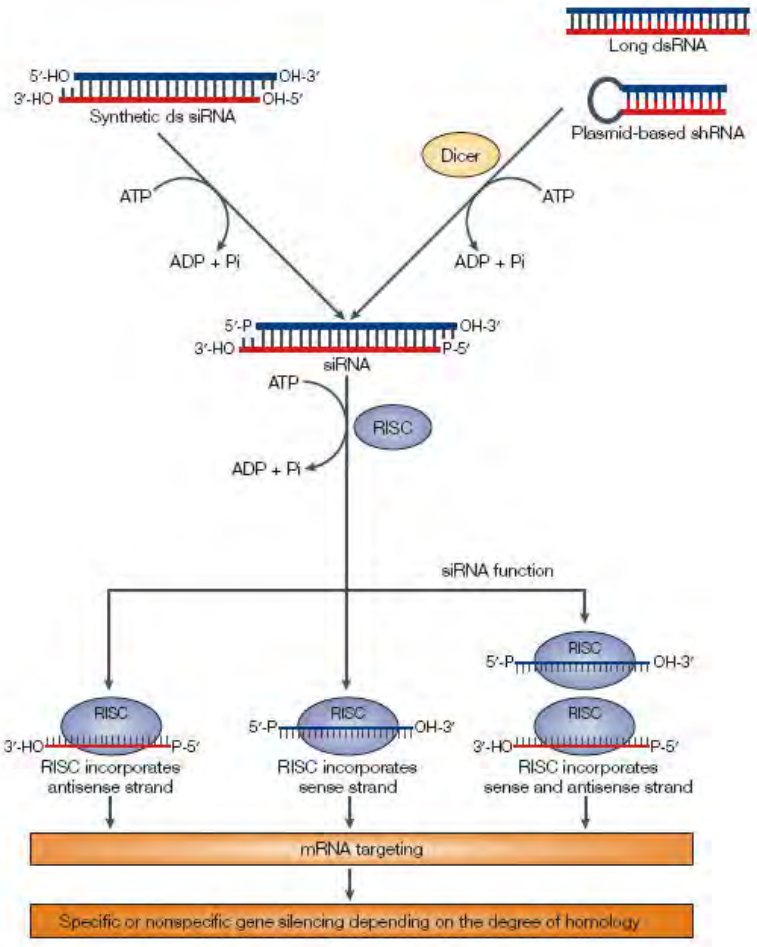

Fig. 1.3 RNAi-mediated gene silencing (Mittal, 2004). The processing of long dsRNA or plasmid-synthesized shRNA by Dicer leads to the formation of siRNAs, which consists of 21- to 23-nt RNA duplexes with symmetric 2-nt 3' overhangs and 5' phosphate groups. Exogenously provided synthetic siRNAs are converted into active functional siRNAs by an endogenous kinase that provides 5' phosphate groups in the presence of ATP. siRNAs associate with cellular proteins to form an RNA-induced silencing complex (RISC), which contains a helicase that unwinds the duplex siRNA in an ATP-dependant reaction. In an ideal situation, the antisense strand guides the RISC to the target mRNA for endonucleolytic cleavage. In theory, each of the siRNA strands can be incorporated into RISC and direct RNAi. The antisense strand of a siRNA can direct the cleavage of a corresponding sense RNA target, whereas the sense strand of a siRNA can direct the cleavage of an antisense target.

1.7.2 Assembly of siRNA into RNA silencing effector complexes and mRNA degradation

In the effector step of RNAi, siRNA-containing ribonucleoprotein particles (RNPs) are assembled into a multi-component nuclease, which guides the sequence-specific recognition of the target mRNA (Hammond *et al.*, 2000; Martinez and Tuschl, 2004). This complex is referred to as the RNA-induced silencing complex (RISC). Several forms of RISC, differing in molecular weight and composition, have been reported. The molecular weight ranges between 130-500 kDa and these differences may be due to weak and/or transient association of proteins involved in the initial processing of dsRNA (Dicer), as well as other factors of unknown function (Jaronczyk *et al.*, 2005). Nevertheless, every RISC contains a member of the Argonaute protein family, which is characterized by the presence of a PAZ domain and a carboxy-teminal PIWI domain (Cerutti *et al.*, 2000; Carmell *et al.*, 2002).

Analysis of the ATP requirements revealed that the formation of RISC on siRNA duplexes requires ATP, but once formed, RISC can mediate robust, sequence-specific cleavage of its target in the absence of ATP (Nykänen *et al.*, 2001; Bernstein *et al.*, 2001). Thus, ATP is most likely required for the energy-driven unwinding of siRNA duplexes and other conformational requirements. In this regard, the DEAD-box RNA helicase Armitage has been shown to be required for RISC assembly of *Drosophila melanogaster* (Tomari *et al.*, 2004). It has also been reported that the sequence composition of the siRNA duplexes may have an influence on the ratio of sense and antisense strands that enters the RISC complex (Khvorova *et al.*, 2003; Schwarz *et al.*, 2003). The strand bias has been proposed to be a consequence of a rate-limiting step during the unwinding step that allows the 5' end of the strand positioned at the weakly paired end of the siRNA to enter the RISC first (Khvorova *et al.*, 2003). Structural studies of the PIWI domain of an Argonaute protein from *Archaeoglobus fulgidus*, in association with a siRNA-like duplex, revealed that PIWI specifically recognizes the 5' end of a siRNA and unwinds the first base pair of the duplex (Parker *et al.*, 2005). This suggests that binding to the PIWI domain might be the first step in siRNA duplex unwinding and that PIWI may thus also participate in strand selection. Moreover, recent biochemical and structural studies have indicated that the PAZ domain of Argonaute proteins is a RNA-binding domain that specifically recognizes the terminus of the base-paired helix of siRNA duplexes, including the 2-nt overhang (Lingel *et al.*, 2004; Ma *et al.*, 2004). This stable interaction may ensure the transfer of siRNA from the RNA complex

into RISC by minimizing the possibility of unrelated RNA-processing or RNA-turnover products entering the RNA silencing pathway.

Following assembly of the RNA silencing effector complexes, the single-stranded RISC guides sequence-specific degradation of complementary mRNAs (Martinez *et al.*, 2002a; Martinez and Tuschl, 2004), and Mg^{2+} ions have been shown to be required for this reaction (Schwarz *et al.*, 2004). The cleavage sites for target mRNA are located in the middle of the region spanned by the siRNA duplexes, precisely 10 nt upstream of the target position complementary to the 5' most nucleotide of the guide siRNA (Elbashir *et al.*, 2001b; Martinez *et al.*, 2002a). 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).

Initially, the identification of the catalytic subunit of RISC appeared to be elusive. Dicer is most likely not part of the RISC, because RISC and Dicer activity can be separated and RISC is unable to process dsRNA to siRNA (Hammond *et al.*, 2000). Recent X-ray crystallography analyses of an Argonaute protein from the archaeobacterium *Pyrococcus furiosus* have shown that the PIWI domain is highly similar to that of members of the RNase H family, which are known to cleave the RNA strand of RNA/DNA duplexes (Song *et al.*, 2004). The structural similarity extends also to inclusion of carboxylate residues within the PIWI domain that are thought to be involved in the positioning of a catabolically important divalent metal ion. Since every RISC contains a member of the Argonaute family of proteins, it has been proposed that Argonaute proteins themselves might act as the catalytic subunit, termed Slicer. Experimental evidence to this effect has been provided by Liu *et al.* (2004a), whom indicated that despite siRNAs being capable of binding to all four versions of the human Argonaute proteins, only Argonaute2 was associated with cleavage of target mRNAs. Subsequent site-directed mutagenesis of the carboxylate residues proposed to bind a catalytic metal ion resulted in blocking cleavage of target mRNAs without affecting Argonaute2 expression or siRNA binding. Similarly, both Parker *et al.* (2005) and Ma *et al.* (2005) also reported that mutagenesis of the putative divalent metal ion-binding site located in the PIWI domain ablated Slicer activity.

1.8 RNAi AS A RESEARCH TOOL IN MAMMALIAN CELLS

Despite its utility in diverse organisms, it was initially difficult to detect potent and specific RNAi by dsRNA longer than 30 bp in commonly used mammalian cell culture systems (McManus and Sharp, 2002; Agrawal *et al.*, 2003). This was largely due to the fact that introduction of the dsRNA into the cytoplasm of mammalian cells triggered an interferon (IFN) response, which results in a systemic, non-specific shutdown of protein synthesis (Manche *et al.*, 1992; Stark *et al.*, 1998). Predominant amongst the responses triggered is activation of the dsRNA-activated protein kinase (PKR), a kinase that is activated by dimerization in the presence of dsRNA. Activated PKR stalls translation by phosphorylation of the translation initiation factor eIF2α, causing a non-specific translational shutdown (Manche *et al.*, 1992). In addition, dsRNA also activates 2',5'-oligoadenylate synthetase (2',5'-AS), the product of which is an essential co-factor for a non-specific ribonuclease, RNase L (Minks *et al.*, 1979). The active RNase L 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).

These sequence-nonspecific effects, however, have been resolved when it was reported 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 (Caplen *et al.*, 2001; Elbashir *et al.*, 2001a). 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 (refer to Section 1.7.1). Consequently, this discovery has opened the door to RNAi approaches in mammalian cells, albeit that the gene-specific silencing is transient. In essence, application of RNAi to gene silencing in mammalian systems necessitates that consideration be given to the selection and design of siRNAs, synthesis and delivery of siRNAs, as well as monitoring of the efficiency of gene silencing. These aspects will therefore be discussed in greater detail in the following sections.

1.8.1 siRNA design

At present, it is not possible to predict with complete certainty the degree of gene silencing a particular siRNA will produce and the design of an effective siRNA is still an empirical process. However, a number of general guidelines and recommendations have been made that may increase the probability of producing an effective siRNA (Elbashir *et al.*, 2001a; Mittal,

2004; Reynolds *et al.*, 2004). In addition, high-throughput screening of potential siRNAs and analyses of effective and ineffective siRNAs, to evaluate the influence of a number of features on siRNA potency and specificity, have led to the development of several siRNA design algorithms (Reynolds *et al.*, 2004; Takasaki *et al.*, 2004; Ui-Tei *et al.*, 2004; Taxman *et al.*, 2006). These guidelines and algorithms are, however, only predictive and do not guarantee a gene silencing effect. Nevertheless, based on results of these analyses, it has emerged that the two most important factors influencing siRNA efficiency are the structural characteristics of the siRNA and the target site within the gene.

siRNA duplexes composed of two 21-nt RNA strands, forming a 19-nt base-paired region with 2-nt 3' overhangs, have been found to be optimal for mediating RNAi in mammalian cell cultures (Elbashir *et al.*, 2001a). Subsequently, it has been reported that RNAi can also be induced in mammalian systems with a wide variety of dsRNA agents such as 18-27 bp siRNA duplexes (Caplen *et al.*, 2001; Elbashir *et al.*, 2002; Kim *et al.*, 2005) with or without 2-nt overhangs (Czauderna *et al.*, 2003) or 19-29 bp short hairpin RNAs (shRNAs) (Harborth *et al.*, 2003). Despite siRNAs produced by Dicer cleavage containing 5' phosphate and 3' hydroxyl termini (Nykänen *et al.*, 2001; Elbashir *et al.*, 2001b), phosphorylation of the 5' ends, however, does not seem to be crucial for cell culture applications (Elbashir *et al.*, 2002; Harborth *et al.*, 2003; Amarzguioui *et al.*, 2003) since free 5' hydroxyl groups of RNA molecules are rapidly phosphorylated by endogenous kinases (Schwarz *et al.*, 2003; Martinez *et al.*, 2002a).

RISC activity is dependant on the sequence complementarity between the siRNA strand residing in the RISC and the cytoplasmic mRNA. The effect of mismatches depends on their nature and position with respect to the cleavage site. The presence of single mismatches in the middle of the guide siRNA is more inhibitory that mismatches at the ends of the siRNA strand (Bitko and Barik, 2001; Harborth *et al.*, 2003; Bohula *et al.*, 2003). Moreover, mismatches close to the 5' end of the antisense siRNA are more disruptive than those at the 3' end (Amarzguioui *et al.*, 2003; Jackson *et al.*, 2003), possibly as a consequence of the 5' end of the guide siRNA serving as a "ruler" to define the position of mRNA cleavage (Elbashir *et al.*, 2001b; Martinez *et al.*, 2002a). In contrast, the sequence of the antisense 3' overhang does not play a significant role in target recognition (Elbashir *et al.*, 2001b).

The overall G+C content of a siRNA sequence does not appear to influence silencing efficiency, since duplexes with 30 to 80% G+C content effectively silenced various different target genes (Elbashir *et al.*, 2002). Since the helicase activity involved in separating siRNA strands appears to preferentially unwind weak base pairs (A-U or U-A) rather that strong base pairs (G-C or C-G), and considering that the siRNA strand for which the 5' end is most readily unwound preferentially accumulates into RISC, it therefore follows that the relative strength of base pairing at the 5' end of either siRNA strand partially determines which strand will be incorporated into RISC (Schwarz *et al.*, 2003; Khvorova *et al.*, 2003). Consequently, it has been suggested that siRNAs should be designed to have a lower G+C content at the 5' end of the antisense strand than the 5' end of the sense strand (Schwarz *et al.*, 2003). Application of these recommendations may therefore increase the likelihood of incorporating the antisense strand, corresponding to the target mRNA transcript, into RISC and thus minimize off-target effects caused by the sense strand (Jackson *et al.*, 2003; Ui-Tei *et al.*, 2004).

Several siRNAs synthesized against different regions of the same target mRNAs have been reported to silence gene expression to different extents (Harborth *et al.*, 2001; Vickers *et al.*, 2003; Lambeth *et al.*, 2007). Therefore, structural features of the target mRNA should also be taken into account when designing siRNAs for gene silencing. In general, the target site should be located at least 100 to 200 nt from the AUG initiation codon and within 50 to 100 nt from the termination codon in order to avoid interference by RNA-regulatory proteins that bind to the 5 and 3' untranslated regions (UTRs). It also follows that based on the sequence-specific nature of RNAi, the target site within the mRNA must be accessible to intermolecular base pairing interactions with the siRNA. Prediction of the RNA secondary structure with software may (Kretschmer-Kazemi Far and Sczakiel, 2003) or may not (Harborth *et al.*, 2003; Holen *et al.*, 2002; Xu *et al.*, 2003) correlate with the potency of a siRNA. Therefore, it has been advised that two or more different siRNA duplexes targeting the same mRNA should be independently tested for a reproducible phenotype (Elbashir *et al.*, 2002). For monitoring and validation of the silencing effect, standard molecular biology techniques are used. Whereas methods for quantification of mRNA levels include Northern blot hybridization, quantitative reverse transcriptase (RT)-PCR or real-time PCR, immunodetection methods, such as Western blot analysis, immunoprecipitation and flow cytometry, are used to quantify the level of protein (Elbashir *et al.*, 2002; Sandy *et al.*, 2005).

1.8.2 Strategies for synthesizing siRNAs

The demonstration by Elbashir *et al.* (2001b) that synthetic dsRNAs resembling naturally produced siRNAs can induce sequence-specific gene silencing after transfection into mammalian cells has paved the way for practical gene silencing in mammalian cells. There are currently five different ways in which RNAi can be induced and these comprise of chemical synthesis of siRNA (Caplen *et al.*, 2001; Elbashir *et al.*, 2002), *in vitro* transcription of dsRNA (Donze and Picard, 2002; Capodici *et al.*, 2002), production of siRNAs by *in vitro* digestion of dsRNAs with recombinant Dicer or RNase III (Yang *et al.*, 2002; Yang *et al.*, 2004; Xuan *et al.*, 2006), expression of siRNA in cells by PCR-derived siRNA expression cassettes (Castanotto *et al.*, 2002; Scherer *et al.*, 2004), and expression of siRNAs in cells by plasmid or viral vectors (Brummelkamp *et al.*, 2002a, 2002b; Paddison *et al.*, 2002; Wadhwa *et al.*, 2004). Amongst these, the development of plasmid and viral vectors that allow delivery of RNAi effector molecules into mammalian cells has received considerable attention. In addition to providing a low-cost alternative to chemically synthesized siRNAs, the vector-based strategy is capable of mediating stable target gene inhibition, thus allowing gene function analyses over an extended period of time. Since vector-based RNAi is closely related to the aims of this investigation, it will subsequently be discussed in greater detail.

1.9 VECTOR-BASED EXPRESSION OF RNAi EFFECTOR MOLECULES

1.9.1 Plasmid vectors

Although chemically synthesized siRNAs represent the "gold standard" for RNAi applications, down-regulation of gene expression mediated by siRNAs is transient and typically lasts for three to five days in cell culture (Holen *et al.*, 2002; Krisielow *et al.*, 2002). In an effort to induce long-term gene silencing, different plasmid vectors have been constructed to continually express RNAi effector molecules in transfected mammalian cells. For this purpose, RNA polymerase III promoters such as the mouse or human small nuclear RNA U6 or human RNaseP H1 promoters are typically used to drive expression of the RNAi effector molecules *in vivo*. Not only are these promoters active in all cell types, but they also express large amounts of small RNA molecules from a defined transcription initiation site, resulting in transcripts that are neither capped nor polyadenylated at their 5' and 3' ends, respectively. Moreover, the RNA polymerase III transcription termination site consists of a

stretch of four to six thymidines and results in the addition of two uridines at the 3'-end of the transcribed RNAs (Paule and White, 2000).

Plasmid vector-based RNAi typically relies on RNA polymerase III-mediated transcription of short hairpin RNA (shRNA) structures with a stem of 19-29 bp and a short loop of 3 to 9 nt that are processed intracellularly by Dicer to yield siRNA-like molecules (Brummelkamp *et al.*, 2002a; Paddison *et al.*, 2002; Sui *et al.*, 2002; Yu *et al.*, 2003; Wu *et al.*, 2005). Thus, for expression of an 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. In an alternative approach, U6 promoters placed in tandem (Lee *et al.*, 2002) or on two separate plasmids (Miyagishi and Taira, 2002; Yu *et al.*, 2002) have been used to direct transcription of a sense and antisense strand of a small RNA with a 19-nt gene targeting sequence and 3' uridine overhangs. These transcripts subsequently anneal within the cell to form structures identical to siRNAs (Fig. 1.4).

1.9.2 Viral vectors

A shortcoming of using plasmid vectors for expression of RNAi effector molecules is that they are difficult to introduce into certain cells, *e.g.*, primary cells, and since the plasmids do not integrate into host genomes, it is not possible to create stable shRNA-expressing cell lines. To overcome these obstacles, viral vectors such as adenoviruses, retroviruses and lentiviruses have been developed in which shRNA expression is driven by either RNA polymerase III or CMV transcription units (Xia *et al.*, 2002; Wadhwa *et al.*, 2004).

Adenoviral vectors are capable of infecting many different cell types via receptor-mediated infection through the widely expressed coxsackievirus and adenovirus receptor (CAR), as well as the αvβ1 integrin receptor (Levy *et al.*, 1994). In addition, adenoviruses do not integrate into the host genome and they have a high level of RNA expression that is independent of the cell cycle, making their use for both dividing and non-dividing cells possible. Consequently, adenoviral vectors have been used to transiently express shRNAs in both cells and tissues (Xia *et al.*, 2002; Grimm *et al.*, 2005; Chen *et al.*, 2006). Although retroviral vectors are also able to infect a range of dividing and non-dividing cells, they are, unlike adenoviruses, able to integrate exogenous genes into the genome of host cells. As a

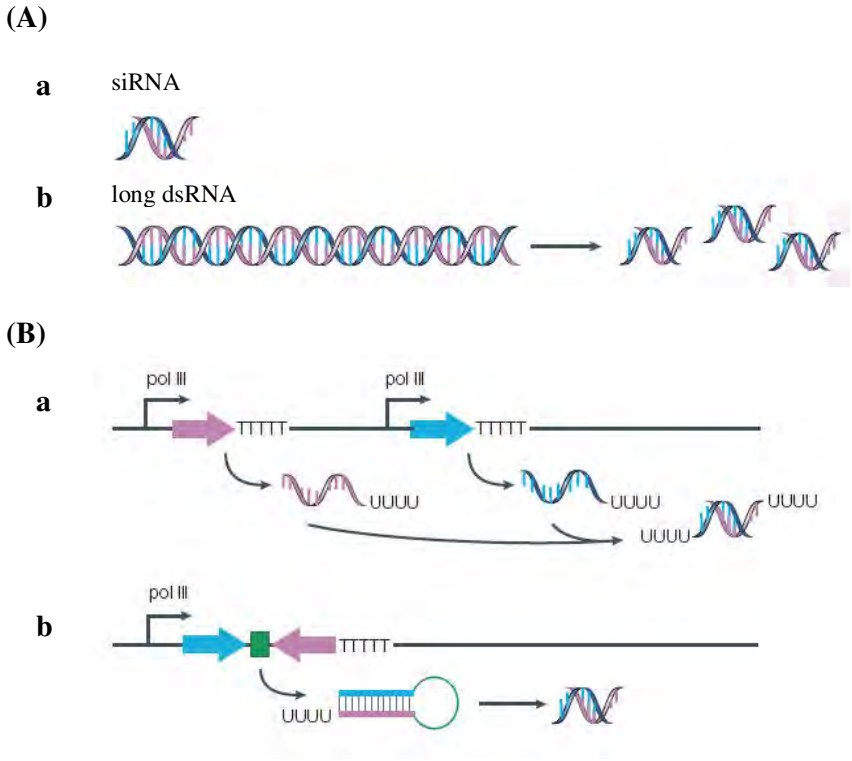

Fig. 1.4 Methods whereby dsRNA can be generated for silencing of gene expression in mammalian cells (Dykxhoorn *et al.*, 2003). RNAi effector molecules can be generated *in vitro* (A) or *in vivo* (B). In *in vitro* production, siRNAs are synthesized chemically (Aa) and following their introduction into mammalian cells they are incorporated into the RNA-induced silencing complex (RISC) for targeted mRNA degradation. Alternatively, long dsRNAs produced by *in vitro* transcription are treated with recombinant Dicer or RNase III (Ab) and the mixture of siRNAs generated is introduced into mammalian cells. (B) In *in vivo* production of RNAi effector molecules, siRNAs can be expressed by tandem polymerase III promoters, which express individual sense and antisense strands of the siRNA, that subsequently associate in *trans* (Ba) or by a single polymerase III promoter that expresses shRNA (Bb), which is processed into a siRNA-like molecule by Dicer cleavage.

result, both retroviral vectors (Brummelkamp *et al.*, 2002b; Liu *et al.*, 2004b; Musiyenko *et al.*, 2007) and replication-deficient lentiviral vectors (Rubinson *et al.*, 2003; Qin *et al.*, 2003) have been used to integrate shRNA expression cassettes into the genome of host cells to create stable shRNA-expressing cell lines. However, integration of the vector may cause disruptions to host genes at the insertion site and there is a possibility that mutations might occur in the shRNA expression cassette during reverse transcription of the retroviral genome by the error-prone viral reverse transcriptase (Hacein-Bey-Abina *et al.*, 2003).

1.9.3 Inducible and conditional expression of RNAi effector molecules

Although plasmid- and viral-based constitutive expression of shRNAs frequently results in effective suppression of target genes, a major limitation of such unregulated RNAi technologies is that it cannot be applied to study the functions of essential genes. To overcome this obstacle, a number of inducible and conditional RNAi systems have been developed.

Tetracycline-inducible H1 (Van de Wetering *et al.*, 2003) and U6 (Matsukura *et al.*, 2003) promoters have been constructed by replacement of a 19-bp sequence between the TATA box and the transcription start site with a tetracycline-doxycycline response element (*tetO*), which serves as a binding site for the *tet* repressor. In transgenic cells expressing the repressor, repressor binding to the *tetO* site blocks transcription, while addition of doxycycline to the culture medium results in dissociation of the repressor, allowing transcription to proceed. Using a similar strategy, an inducible system utilizing the *lac* repressor has been described (Higuchi *et al.*, 2004). In this case, an *E. coli lacO* operator element was used to replace a 26-nt sequence between the TATA box and the transcription initiation start site in the H1 promoter, thus resulting in IPTG-responsive expression in transgenic cells expressing the *lac* repressor. In both systems, re-emergence of target gene expression has been demonstrated to occur within three to four days of withdrawing the inducer. This delay is though to be due to the relatively long half-life of shRNAs (Matsukura *et al.*, 2003).

In addition to the above, a three-component ecdysone-inducible retroviral system has been developed (Gupta *et al.*, 2004). In this system, two vectors are used that encode the nuclear receptors/transcription factors, *i.e.* the chimeric ecdysone receptor (VgECR) and retinoid X receptor (RXR). The third vector contains a GAL4-Oct-2^{Q} chimeric transactivator under the

control of the ecdysone-inducible promoter and a modified U6 promoter with four GAL4-binding sites in place of the enhancer. Addition of the ecdysone analogue muristeroneA initiates an activation cascade involving dimerization of VgECR and RXR, which, in turn, activates the GAL4-Oct-2^{Q} transactivator expression and finally, activation of the modified U6 promoter and transcription of the shRNA. Similar to the doxycycline-inducible U6 and *lac*-responsive H1 expression systems, shRNA expression was repressible and target gene expression recovered gradually from two to four days after withdrawal of the inducer (Gupta *et al.*, 2004).

For conditional shRNA-mediated suppression of gene expression, different Cre-LoxP recombination-based systems have been reported (Kasim *et al.*, 2004; Tiscornia *et al.*, 2004; Ventura *et al.*, 2004). The technology involves the insertion of a loxP-flanked stuffer fragment between either the distal sequence element (DSE) and proximal sequence element (PSE) or the PSE and TATA box element within the U6 promoter. Introduction of Cre activity by transduction of cells with recombinant viral vectors expressing the Cre recombinase results in recombination between the two loxP sites and removal of the intervening stuffer fragments. This allows for the different elements of the promoter to be brought together, thereby generating an active promoter (Tiscornia *et al.*, 2004; Ventura *et al.*, 2004). However, in contrast to the above-mentioned inducible RNAi systems, the Cre-loxP system involves genomic rearrangements and is therefore irreversible.

1.10 RNAi IN VIROLOGY: APPLICATIONS AND CONSTRAINTS

Since the initial report by Elbashir *et al.* (2001a), whom identified RNAi activity in mammalian cells, many publications have subsequently described the use of RNAi to inhibit viruses from diverse virus families. Genes that are essential for viral replication have especially become a focus in the development of RNAi as an antiviral principle. In general, either synthetic siRNAs or vector-expressed shRNAs have been used, while a few reports have used long dsRNA to mediate silencing of target viral genes. Despite the success already achieved, there are some constraints that also need to be taken into account in the development of effective RNAi-based antiviral therapies.

1.10.1 Applications

Inhibition of viral genome transcription after entering the host cell or inhibition of genes that encode proteins that play an essential role during genome replication, are strategies frequently used to inhibit virus replication in infected mammalian cells. By making use of 500-nt long dsRNAs that targeted the Gag and Env structural proteins of HIV-1, Park *et al.* (2002) reported that virus replication was inhibited 70-fold. In contrast, siRNA-mediated silencing of the Pol enzyme (Hu *et al.*, 2002) or the Tat and Rev regulatory proteins (Coburn and Cullen, 2002) was reported to inhibit virus replication 10-fold in both instances, whereas intracellularly-expressed shRNAs directed against the two accessory proteins Nef and Vif, respectively, were reported to inhibit virus replication 20-fold (Jacque *et al.*, 2002). More recently, Ge *et al.* (2003) reported that siRNAs targeted at the two proteins of the transcriptase complex (PA and PB1) of influenza A virus, resulted in the overall inhibition of viral transcription in mammalian cell culture, whereas Hui *et al.* (2004) reported that siRNA directed against the virus matrix gene was equally effective in inhibiting virus replication in 293T cells.

RNAi has also been used to inhibit the expression of viral attachment proteins and cellular membrane molecules that act as viral receptors, thereby preventing viral entry by blocking attachment of viruses to surface receptors and thus providing some form of virus resistance. In this regard, siRNA-mediated silencing of the genes encoding CD4, the main receptor for HIV-1 (Novina *et al.*, 2002), and of both CCR5 and CXCR4, which are co-receptors for infection by most strains of HIV-1 (Martinez *et al.*, 2002b), were reported to result in a 4-fold and 2-fold reduction in HIV-1 entry, respectively. However, use of a lentiviral-based vector system to introduce shRNA against CCR5 in peripheral blood T-lymphocytes resulted in a 3- to 7-fold decrease in the number of infected cells (Qin *et al.*, 2003). In an alternative approach to targeting viral receptors, the fusion protein of respiratory syncytial virus (Bitko and Barik, 2001) and spike protein of severe acute respiratory syndrome-associated caronavirus (SARS-CoV) (Zhang *et al.*, 2004b; Wu *et al.*, 2005), which play important roles in viral entry and pathogenesis, have been targeted for silencing. Silencing expression of these viral attachment proteins by siRNAs and a shRNA-expressing plasmid vector, respectively, resulted in significant reductions in virus titre in cell cultures.

In addition to the above, RNAi has also been used to hinder the proper assembly of viral particles by silencing expression of viral capsid protein-encoding genes. Capsid proteins have become favourable targets for degradation, since they have essential functions in the virus replication cycle, amongst other, mediating attachment of virus particles to cell receptors, facilitating penetration of virions into the cell and are required for the assembly of virus particles prior to their release from infected cells (Levy et al., 1994). In the case of rotavirus (Déctor *et al.*, 2002) and AHSV-9 (Stassen *et al.*, 2007), it has been reported that siRNAs targeting VP4 and VP7, respectively, reduced the yield of infectious progeny. Similarly, Gitlin *et al.* (2002) reported that siRNA directed against the P1-encoding region on the poliovirus genome, which encodes the viral capsid proteins VP1 to VP4, prevented poliovirus infection in mammalian cell. Moreover, siRNAs directed at the VP1 (Chen *et al.*, 2004) and VP4 (Liu *et al.*, 2005) capsid-encoding genes of foot-and-mouth disease virus (FMDV) inhibited the virus yield by 50-fold.

1.10.2 Constraints

Although the above highlights the potential of RNAi as a novel antiviral therapeutic agent, there are, however, a number of constraints that would need to be addressed in order for it to reach its full potential as an antiviral approach. In addition to concerns regarding the delivery and efficacy of different siRNAs, it has been reported that some viruses encode suppressors of the RNAi pathway. The B2 protein encoded by flockhouse virus (FHV), the E3L protein encoded by vaccinia virus and the NS1 protein encoded by influenza virus have all been reported to suppress RNAi in *Drosophila* insect cells (Li *et al.*, 2002; Li *et al.*, 2004), albeit through an as yet unknown mechanism. Lichner *et al.* (2003) reported that the dsRNA-binding protein σ3 of reovirus suppressed RNAi in plants, possibly through the sequestering of dsRNAs. In contrast to the above, both adenovirus (Lu and Cullen, 2004) and nodamura virus (NoV) (Sullivan and Ganem, 2005) have been reported to encode suppressor proteins that function in mammalian cells. Whereas it was shown that the adenovirus VA1 RNA binds to Dicer and subsequently inhibits its function, the NoV B2 protein was shown to bind to both the pre-Dicer substrate RNA and RISC-processed RNA, thus suggesting that it inhibits the Dicer cleavage reaction and post-Dicer activities.

A specific concern regarding RNA viruses and retroviruses, which exhibit significant genetic variation due to an error-prone replication mechanism, is the possibility of viral escape. Since

a limited number of mismatches between the siRNA and target RNA may abolish the suppressive action of the siRNA (Gitlin *et al.*, 2002; Elbashir *et al.*, 2001b; Martinez *et al.*, 2002a), it can be expected that escape mutants can arise during each cycle of the virus replication that will be resistant to RNAi. Indeed, RNAi-resistant poliovirus (Gitlin *et al.*, 2002), HIV-1 (Das *et al.*, 2004; Westerhout *et al.*, 2005) and hepatitis C (Wilson and Richardson, 2005) viruses have been reported, and it was shown that these viruses overcame RNAi inhibition through the accumulation of several point mutations within the siRNA target sequence (Wilson and Richardson, 2005) or by modifying the target sequence though nucleotide substitutions and deletions (Gitlin *et al.*, 2002; Das *et al.*, 2004). To circumvent the emergence of escape mutants, it has been recommended that multiple different siRNAs should be used that target highly conserved regions of the viral genome (Liu *et al.*, 2005; Wilson and Richardson, 2005). Alternatively, single (Novina *et al.*, 2002) or multiple (Qin *et al.*, 2003) host cell factors such as receptors that are involved in viral replication may also be targeted for gene silencing in an effort to limit the possibility for viral escape.

It is also possible that virus replication strategies may shield the viral genome from the RNAi machinery, thus protecting the viral genomic RNA from degradation. In this regard, Silvestri *et al.* (2004) reported that despite being able to silence rotavirus VP7 and VP4 protein expression, viral particles that contained all 11 dsRNA genome segments in equimolar amounts were still formed in both instances. Based on these results, it was proposed that rotavirus positive-sense mRNAs that are located outside of the viroplasms and undergo translation are susceptible to siRNA-induced degradation, whereas positive-sense mRNAs that are located inside of the viroplasms and undergo replication are not susceptible to degradation. Thus, it would appear that association of the viral transcripts with the viroplasm, formed by the non-structural protein NSP5, may render them inaccessible to the RNAi machinery. Similarly, the inability to inhibit AHSV-9 virus replication by siRNA-mediated silencing of VP7 protein expression (Stassen *et al.*, 2007), suggests that the formation of VIBs during AHSV replication may present a similar challenge for the RNAi machinery. Although the use of RNAi as an antiviral therapeutic tool for this family of viruses remains to be demonstrated, the results obtained from the above studies suggest that RNAi may provide for new experimental approaches whereby viral gene function can be investigated *in vivo*.

1.11 AIMS OF THIS INVESTIGATION

From the review of the literature, it is evident that many details regarding orbivirus replication, morphogenesis and assembly still need to be elucidated. The non-structural proteins are thought to play an important role in these processes, especially the NS2 protein. The NS2 protein is phosphorylated, capable of binding ssRNA and is closely associated with the formation of VIBs, which are the sites in which virus morphogenesis occurs (Hyatt and Eaton, 1988; Kar *et al.*, 2007). A major constraint to the investigation of orbivirus genes has been the lack of a reverse genetics system and consequently, characterization of the viral genes has been limited to their expression in prokaryotic or eukaryotic expression systems (Vreede and Huismans 1994; Uitenweerde *et al.*, 1995; van Niekerk *et al.*, 2001; de Waal and Huismans, 2005). However, RNAi technology has emerged as a potentially powerful tool for reverse genetics studies in organisms where generating loss-of-function phenotypes have proven difficult or impossible (McManus and Sharp, 2002; Elbashir *et al.*, 2002). Due to the segmented nature of orbivirus genomes, it is possible to silence expression of specific individual genes without affecting the expression of the other viral genes (Stassen *et al.*, 2007). Analysis of such loss-of-function phenotypes may facilitate greatly investigations regarding virus gene function in the context of virus-infected cells, a task that has been elusive thus far. RNAi is most commonly achieved by transfecting synthetic siRNAs into mammalian cells (Caplen *et al.*, 2001; Elbashir *et al.*, 2001a; Elbashir *et al.*, 2002). However, the high cost of siRNA synthesis and transient nature of the gene silencing are limiting factors for its general use. More recently, several plasmid DNA-based vectors have been developed that direct transcription of shRNAs, which are processed intracellularly into functional siRNAs by Dicer (Brummelkamp *et al.*, 2002a; Paddison *et al.*, 2002; Wu *et al.*, 2005). Not only is this approach a cost-effective alternative to the use of synthetic siRNAs, but it also purportedly allows for stable gene silencing over an extended period of time. Therefore, towards the long-term goal of investigating the *in vivo* functional role of NS2 during virus replication and morphogenesis, the specific aims of this investigation were as follow:

- To construct and characterize an AHSV-9 NS2-eGFP chimeric gene for use in the screening of different candidate NS2-directed shRNAs.

- To develop a plasmid DNA-based RNAi assay whereby expression of the AHSV-9 NS2 gene could be silenced by shRNA in BHK-21 cell culture.

CHAPTER TWO

MAMMALIAN AND BACULOVIRUS EXPRESSION OF AN AHSV-9 NS2-eGFP CHIMERIC GENE

2.1 INTRODUCTION

African horse sickness virus (AHSV) is a member of the *Orbivirus* genus in the family *Reoviridae*. Like other orbiviruses, the genome of AHSV consists of 10 double-stranded (ds)RNA segments that encode seven structural (VP1-VP7) and four non-structural (NS1, NS2, NS3/NS3A) proteins (Bremer *et al.*, 1990; Grubman and Lewis, 1992). It is believed that the non-structural proteins are involved in the process of viral morphogenesis, leading to viral assembly and release (Roy *et al.*, 1994b; Roy, 2001). The non-structural protein NS2 may play a key role in these processes due to its ability to bind single-stranded (ss)RNA (Huismans *et al.*, 1987b; Uitenweerde *et al.*, 1995; Theron and Nel, 1997; Lymperopoulos *et al.*, 2006) and it being the major component of virus inclusion bodies (VIBs) formed in virus-infected mammalian cells (Eaton *et al.*, 1987; Brookes *et al.*, 1993).

The focus of our group's research is to study the *in vivo* functional role of AHSV-9 non-structural proteins in virus replication. These types of studies have been hampered due to a lack of a suitable reverse genetic system for orbiviruses. However, it has been demonstrated that RNA interference (RNAi) can be used for gene silencing and it has subsequently been used widely for functional studies (McManus and Sharpe, 2002; Means *et al.*, 2003; Ikeda *et al.*, 2004; Campagna *et al.*, 2005; Wirblich *et al.*, 2006; Forzan *et al.*, 2007). RNAi is an evolutionary conserved mechanism by which RNA duplexes, known as short interfering RNA (siRNA), can reduce gene expression through enzymatic cleavage of complementary mRNA (Fire *et al.*, 1998; Elbashir *et al.*, 2001b; Meister and Tuschl, 2004). The segmented nature of the AHSV genome makes it particularly amenable to analysis by RNAi, since each of the 10 dsRNA genome segments is transcribed into a single mRNA, each encoding a single protein (except for NS3). This therefore makes it possible to silence the expression of individual virus genes without affecting the expression of others (Silvestri *et al.*, 2004; Stassen *et al.*, 2007).

RNAi is commonly achieved by introducing chemically synthesized siRNA into cells or, alternatively, by short hairpin RNA (shRNA) that is expressed intracellularly from plasmid and viral vectors (Agrawal *et al.*, 2003; Wadhwa *et al.*, 2004; Amarzguioui *et al.*, 2005). At present, it is not possible to predict with complete certainty the degree of gene silencing a particular si/shRNA will produce. However, a number of recommendations have been made that may increase the probability of producing an effective siRNA (Elbashir *et al.*, 2001a;

Mittal, 2004; Reynolds *et al.*, 2004; Takasaki *et al.*, 2004; Ui-Tei *et al.*, 2004). Since variation in the silencing efficiency of si/shRNAs targeting the same mRNA has been reported (Harborth *et al.*, 2001; Holen *et al.*, 2002; Vickers *et al.*, 2003; Lambeth *et al.*, 2007), it is therefore useful to evaluate the silencing capability of several candidate si/shRNAs. Moreover, since assays with infectious viruses are time-consuming and labour-intensive, a simple method for the evaluation of active si/shRNAs is required. A number of assay methods have been used to evaluate gene silencing in mammalian cells. These include methods aimed at detecting endogenous mRNA levels of silenced genes such as Northern blot analysis or quantitative reverse transcriptase-PCR (qRT-PCR) (Elbashir *et al.*, 2002; Sandy *et al.*, 2005). However, these methods are expensive to perform and not suited to rapid screening of several different candidate si/shRNAs. Consequently, detection of down-regulation of the endogenous protein levels has become the preferred method for this purpose (Sandy *et al.*, 2005). In addition to performing Western blot analysis with protein-specific antibodies, other approaches have relied on cloning of the target gene's coding sequence in-frame with a small antigen epitope and determining the extent of silencing of the expressed protein with an antibody against the tag (Wu *et al.*, 2004). Instead of a small epitope, fluorescent (*e.g.*, green fluorescent protein [eGFP]) and enzymatic (*e.g.*, luciferase) reporters have also been used as fusion partners (Yokota *et al.*, 2003; de los Santos *et al.*, 2005; Liu *et al.*, 2006; Lantermann *et al.*, 2007). Upon transcription, these constructs provide a chimeric mRNA and efficient targeting of the gene of interest leads to degradation of the mRNA, and therefore no reporter product is produced. Because fluorescence is easy to visualize under a fluorescent microscope and can be quantified by fluorometry or flow cytometry, the eGFP gene has become a popular reporter whereby RNAi can be studied (Sandy *et al.*, 2005).

In this study, initial attempts at expressing an AHSV-9 NS2-eGFP chimeric gene in BHK-21 cells with a mammalian expression vector yielded levels of expression that were not detectable by Western blotting and thus precluded characterization of the chimeric protein prior to its use in si/shRNA screening assays. Consequently, to allow for characterization of the NS2-eGFP protein the baculovirus expression system was selected, since it is one of the most popular and versatile eukaryotic expression systems for heterologous protein expression (O'Reilly *et al.*, 1992; Possee, 1997; Yin *et al.*, 2007). This expression system provides a cellular environment that is conductive to the proper folding, disulfide bond formation, oligomerization and post-translational modification required for biological activity of some proteins (O'Reilly *et al.*, 1992; Yin *et al.*, 2007). Indeed, the synthesis of individual AHSV

proteins by appropriately constructed baculovirus recombinants in *Spodoptera frugiperda* insect cell culture has made significant contributions to the structure-function relationships of several viral proteins, including NS1 (Maree and Huismans, 1997), NS2 (Uitenweerde *et al.*, 1995), NS3 (Van Staden *et al.*, 1995), VP6 (de Waal and Huismans, 2005) and VP7 (Burroughs *et al.*, 1994; Basak *et al.*, 1996). Furthermore, co-infection of *S. frugiperda* cells with VP3 and VP7 recombinant baculoviruses have been reported to result in intracellular accumulation of multimeric particles that resemble authentic AHSV core-like particles (Maree *et al.*, 1998).

Taking the above into consideration, the principal aim of this part of the investigation was to engineer a recombinant bacmid expressing an AHSV-9 NS2-eGFP chimeric gene and to characterize the expressed protein in order for it to be used towards the establishment of a si/shRNA screening assay (Chapter 3).

2.2 MATERIALS AND METHODS

2.2.1 Bacterial strains and plasmids

Escherichia coli strains were 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 plates or at -70°C as glycerol cultures. For plasmid DNA selection and maintenance in *E. coli*, the following concentrations of antibiotics were used: 50 µg/ml for ampicillin, 50 µg/ml for kanamycin, 10 µg/ml for tetracycline and 7 µg/ml for gentamycin. All antibiotics were obtained from Roche Diagnostics and Sigma-Aldrich. Recombinant plasmid pGEX-AHSVNS2, which contains a full-length cDNA copy of AHSV-9 genome segment 8, was obtained from Prof. J. Theron (Department of Microbiology and Plant Pathology, University of Pretoria). Recombinant plasmids pGEM-eGFP and pCMV-NS2, which contain full-length copies of the enhanced green fluorescent (eGFP) and AHSV-9 NS2 genes, respectively, were obtained from Mrs. L. Stassen (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, while the pCMV-Script® mammalian expression vector was obtained from Stratagene.

2.2.2 Plasmid DNA extraction

Plasmid DNA was extracted with the alkaline lysis method (Birnboim and Doly, 1979), as described by Sambrook *et al.* (1989). Single bacterial colonies were inoculated into 5 ml of LB broth

supplemented with the appropriate antibiotic, and cultured overnight at 37°C with shaking. The cells from 3 ml of the overnight culture were collected by centrifugation at 15 000 rpm for 1 min in a 1.5-ml Eppendorf tube. The cell pellet was suspended in 100 µl of ice-cold Solution I (50 mM glucose; 10 mM EDTA; 25 mM Tris; pH 8) and incubated at room temperature for 5 min, followed by 1 min on ice. The cells were lysed by addition of 200 µl of Solution II (0.2 N NaOH; 1% [w/v] SDS) and after incubation on ice for 5 min, 150 µl of ice-cold Solution III (3 M NaOAc; pH 4.8) was added. After incubation on ice for 10 min, the insoluble aggregate that formed was removed by centrifugation at 15 000 rpm for 15 min. The plasmid DNA was precipitated from the recovered supernatant by addition of two volumes of 96% ethanol and incubation at -70°C for 30 min. The plasmid DNA was pelleted by centrifugation at 15 000 rpm for 5 min, rinsed with 70% ethanol, vacuum-dried and suspended in 30 µl of 1 × TE buffer (10 mM Tris-HCl; 1 mM EDTA; pH 8). To remove contaminating RNA, extracted plasmid DNA was incubated at 37°C for 30 min with 0.5 µl of RNase A (10 mg/ml).

2.2.3 DNA amplification

2.2.3.1 Primers

Towards construction of a NS2-eGFP chimeric gene, different primers were used to amplify the full-length eGFP gene and a truncated version of the AHSV-9 NS2 gene. The primers were designed based on the available nucleotide sequence of the AHSV-9 NS2 (GenBank Acc. no. M69090) and eGFP (GenBank Acc. no. AY237157) genes by making use of the DNAMAN v.4.13 (Lynnon Biosoft) software program. To facilitate cloning of the amplicons, unique restriction endonuclease recognition sites were incorporated at the 5'-terminus of the respective primers. The primers, indicated in Table 2.1, were obtained from Inqaba Biotechnical Industries.

2.2.3.2 Polymerase chain reaction (PCR)

Each PCR reaction mixture (50 µl) contained 50 ng of template DNA, 1 × PCR buffer, 1.5 mM $MgCl_2$, 50 pmol of each of the sense and antisense primer, 2.5 mM of each deoxynucleotide triphosphate (dNTP) and 1 U of SUPERTHERM *Taq* DNA polymerase (Southern Cross Biotechnology). The reaction mixtures were placed in a Perkin-Elmer GeneAmp® 2400 thermal cycler. After initial denaturation at 94°C for 3 min, the samples were subjected to 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and elongation at 72°C for 1 min. For control purposes, reaction mixtures were prepared that contained all of the above-mentioned reagents, except the template DNA. Following PCR, aliquots of the reaction mixtures were analyzed by agarose gel electrophoresis in the presence of an appropriate DNA molecular weight marker.

Table 2.1 Primers used in PCR and nucleotide sequencing reactions

Primer	Nucleotide sequence
PCR amplification*:	
BamHINS2-F	5' - CAC**ggatcc**ATGGCAGAGGTCA - 3'; *Bam*HI site incorporated
EcoRINS2-R	5' - CAC**gaattc**ACCGCTCCCCCCT - 3'; *Eco*RI site incorporated
EcoRIeGFP-F	5' - CAC**gaattc**ATGGTGAGCAAGGG - 3'; *Eco*RI site incorporated
XhoIeGFP-R	5' - CAC**ctcgag**TTACTTGTACAGCTCGT - 3'; *Xho*I site incorporated
Nucleotide sequencing:	
pUC/M13-F	5' - TGTAAAACGACGGCCAGT - 3'
pUC/M13-R	5' - CAGGAAACAGCTATGAC - 3'
T3	5' - AATTAACCCTCACTAAAGGG - 3'
T7	5' - GTAATACGACTCACTATAGGG - 3'
eGFP-IR	5' - TCGGGGTAGCGGCTGAAGCAC - 3'

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

2.2.4 Agarose gel electrophoresis

DNA was analyzed by agarose gel electrophoresis. For this purpose, horizontal 1% (w/v) agarose gels were cast and electrophoresed at 100 V in 1 × TAE buffer (40 mM Tris-HCl; 20 mM NaOAc; 1 mM EDTA; pH 8.5). The agarose gels were supplemented with ethidium bromide (0.5 µg/ml) and the DNA visualized by UV fluorescence on a 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 *Eco*RI and/or *Hind*III (Roche Diagnostics). Gel images were captured with a digital gel documentation system (Vilber Lourmat).

2.2.5 Purification of DNA fragments from agarose gels

DNA fragments were purified from agarose gel slices with a silica suspension, as described by Boyle and Lew (1995). The DNA fragment of interest was excised from the agarose gel, placed into a 1.5-ml Eppendorf tube, mixed with 400 µl of a 6 M NaI solution and incubated at 55°C. Following complete dissolution of the agarose, 7 µl of a silica suspension was added to the sample and then incubated on ice for 30 min with intermittent vortexing. The silica-bound DNA was pelleted by centrifugation at 15 000 rpm for 30 s and washed three times with 500 µl of ice-cold New Wash Solution (50 mM NaCl; 10 mM Tris [pH 8]; 2.5 mM EDTA; 50% (v/v) ethanol). The DNA was eluted from the silica matrix at 55°C for 5 min in a final volume of 7 µl of 1 × TE buffer. An aliquot (1 µl) of the eluted DNA was analyzed by agarose gel electrophoresis to assess the concentration and purity of the DNA.

2.2.6 Cloning of DNA fragments into plasmid vectors

2.2.6.1 Ligation reactions

For cloning of PCR amplicons, the pGEM®-T Easy vector system (Promega) was used according to the manufacturer's instructions. Briefly, the gel-purified amplicon (250 ng) and pGEM®-T Easy vector DNA (50 ng) were ligated at 15°C for 16 h in a reaction mixture that contained 5 µl of a 2 × Rapid ligation buffer, 1 µl of T4 DNA ligase (3 U/µl) and UHQ water to a final volume of 10 µl. For all other ligation reactions, the DNA fragments of interest and restricted vector DNA were ligated at 15°C for 16 h in a 10-µl reaction volume, containing 1 µl of a 10 × DNA ligase buffer and 1 µl of T4 DNA ligase (1 U/µl; Roche Diagnostics). The ratio of insert to vector DNA was typically in excess of 3:1.

2.2.6.2 Preparation of competent *E. coli* DH5α cells

Competent *E. coli* DH5α cells were prepared and transformed according to methods described by Sambrook *et al.* (1989). A single colony of a freshly streaked culture of *E. coli* DH5α was inoculated

into 100 ml of LB broth and cultured overnight at 37°C with shaking. An aliquot of the overnight culture was then used to inoculate 100 ml of LB broth in a 250-ml Erlenmeyer flask, pre-warmed to 37°C. The culture was incubated at 37°C with shaking and the optical density (OD) was measured at 550 nm every 30 min until an OD_{550} of 0.4 was reached. The flask was then incubated on ice for 15 min to inhibit further growth. The cells from 30 ml of the culture were collected by centrifugation in a Sorvall® centrifuge at 4 000 rpm for 10 min at 4°C. The cell pellet was suspended gently in 10 ml of ice-cold 0.1 M $CaCl_2$ and incubated on ice for 1 h. The cells were harvested, as described above, and resuspended in 1 ml of the 0.1 M $CaCl_2$ solution. The competent cells were incubated on ice for 1 h prior to transformation or, alternatively, were snap-frozen in 15% (v/v) sterile glycerol and stored at -70°C until further use.

2.2.6.3 Transformation of competent cells

To transform the prepared competent *E. coli* DH5α cells, 200 µl of the competent cells was mixed in a pre-chilled Eppendorf tube with 10 µl of the ligation reaction mixture. After incubation on ice for 30 min, the tubes were subjected to a heat shock by incubating the tubes at 42°C for 90 s in a water bath. After chilling the tubes on ice for 2 min, 800 µl of pre-warmed (37°C) LB broth was added to the tubes followed by incubation at 37°C for 1 h. As controls, competent cells were transformed with DNA of a known concentration to determine the transformation efficiency, and untransformed cells were used to test for contamination. Aliquots (200 µl) of the transformation mixtures were subsequently plated onto LB agar supplemented with the appropriate antibiotic. For plasmids containing a *lacZ'* marker gene (pGEM®-T Easy), the cells were plated together with 50 µl of 2% (w/v) X-Gal and 10 µl of 100 mM IPTG. The agar plates were incubated overnight at 37°C until the colonies were clearly visible.

2.2.7 Restriction endonuclease digestion

Approximately 0.3-1 µg of plasmid DNA was digested with 5-10 U of enzyme in the appropriate concentration of salt (using the 10× buffer supplied by the manufacturer). The reaction volumes were typically 15 µl and incubation was at 37°C for at least 1 h. For digestions involving two enzymes of which the salt concentrations differed for optimal activity, the plasmid DNA was first digested with the enzyme requiring the lowest salt concentration. The salt concentration was then adjusted and the second enzyme added. All the restriction endonucleases were supplied by Roche Diagnostics. Following digestion, the restriction products were analyzed by agarose gel electrophoresis in the presence of an appropriate DNA molecular weight marker.

2.2.8 Nucleotide sequencing

Nucleotide sequencing of cloned insert DNA was performed with the ABI-PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction kit v.3.1 (Perkin-Elmer Applied Biosystems) according to the manufacturer's instructions. The T3 and T7 primers (pCMV-Script®), and universal pUC/M13 forward and reverse primers (pGEM®-T Easy) were used as sequencing primers, in addition to an eGFP-specific internal primer (eGFP-IR) (Table 2.1). The sequencing reactions comprised 250-500 ng of purified template DNA, 2 µl of Terminator Ready Reaction Mix, 10 pmol of the sequencing primer and UHQ water to a final volume of 10 µl. Cycle sequencing reactions were performed in a Perkin-Elmer GeneAmp® 2400 thermal cycler with 25 of the following cycles: denaturation at 96°C for 10 s, annealing at 50°C for 5 s and extension at 60°C for 4 min. The extension products were precipitated by addition of 2 µl of 3 M NaOAc (pH 4.6) and 50 µl of 100% ethanol. Following incubation at room temperature for 15 min in the dark, the tubes were centrifuged at 15 000 rpm for 30 min and the supernatant carefully aspirated. The pellet was washed twice with 70% ethanol, vacuum-dried and stored at -20°C. The purified, denatured extension products were loaded onto a Hitachi 3100 capillary array DNA sequencer. Nucleotide sequences were analyzed with DNAMAN v.4.13 (Lynnon Biosoft) and BioEdit v.5.0.9.1 (Hall, 1999) software programs, and their identity were verified by BLASTN searches (Altschul *et al.*, 1997) against the GenBank database (available at http://www.ncbi.nlm.nih.gov/BLAST).

2.2.9 Plasmid constructions

All molecular cloning techniques used in the construction of the respective recombinant plasmids were performed according to the procedures described in the preceding sections. All plasmid constructs were confirmed by restriction endonuclease digestion and by nucleotide sequencing.

- **pGEM-NS2Trunc**

Primers BamHINS2-F and EcoRINS2-R were used with plasmid pGEX-AHSVNS2 as template DNA to PCR-amplify a truncated NS2 gene lacking a stop codon (1.098 kb). The amplicon was subsequently cloned into pGEM®-T Easy to generate pGEM-NS2Trunc.

- **pGEM-eGFP(M)**

Primers EcoRIeGFP-F and XhoIeGFP-R were used with plasmid pGEM-eGFP as template DNA to generate a 720-bp amplicon, which was cloned into pGEM®-T Easy to yield pGEM-eGFP(M).

- **pCMV-NS2-eGFP**

Towards construction of the recombinant mammalian expression vector pCMV-NS2-eGFP, the truncated NS2 gene was recovered from pGEM-NS2Trunc by digestion with both *Bam*HI and *Eco*RI,

and cloned into identically digested pCMV-Script® to generate pCMV-NS2Trunc. To complete construction of pCMV-NS2-eGFP, the cloned eGFP gene was recovered from pGEM-eGFP(M) by digestion with both *Eco*RI and *Xho*I, and cloned into identically digested pCMV-NS2Trunc.

- **pFB-NS2-eGFP**

To construct the recombinant pFastBac1™ donor plasmid pFB-NS2-eGFP, the 1.818-kb NS2-eGFP chimeric gene was excised from plasmid pCMV-NS2-eGFP by digestion with both *Bam*HI and *Xho*I, and then cloned into pFastBac1™ that had been prepared in an identical manner.

- **pFB-NS2**

To construct the recombinant pFastBac1™ donor plasmid pFB-NS2, the full-length 1.116-kb AHSV-9 NS2 gene was excised from plasmid pCMV-NS2 by digestion with *Bam*HI and then cloned into *Bam*HI-digested plasmid pFastBac1™.

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 kanamycin (50 µg/ml) and tetracycline (10 µg/ml). Competent *E. coli* DH10Bac™ cells were prepared, as described previously (Section 2.2.6.2). For transformation, 500 ng of the recombinant pFastBac1™ donor plasmid DNA was mixed with 100 µl of the competent *E. coli* DH10Bac™ cells in a 1.5-ml Eppendorf tube and then incubated on ice for 30 min. The cells were heat-shocked at 42°C for 45 s and placed on ice for 2 min, followed by addition of 900 µl of pre-warmed (37°C) SOC broth (2% [w/v] tryptone; 0.5% [w/v] yeast extract; 10 mM NaCl; 2.5 mM KCl; 20 mM glucose; 10 mM $MgCl_2$; pH 7). The transformation mixtures were incubated at 37°C for 4 h to allow for transposition to occur. The transformed cells were plated onto LB agar supplemented with kanamycin (50 µg/ml), gentamycin (7 µg/ml) and tetracycline (10 µg/ml), in the presence of 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 selected and re-streaked twice on the same agar medium, as above, to confirm the phenotype.

2.2.10.2 Extraction of recombinant bacmid DNA

Selected colonies were inoculated into 20 ml of LB broth containing antibiotics (50 µg/ml kanamycin; 7 µg/ml gentamycin; 10 µg/ml tetracycline) and cultured overnight at 37°C with agitation. The

recombinant bacmid DNA was isolated by the following protocol that had been developed for isolating high-molecular-weight plasmid DNA and subsequently adapted for isolating recombinant bacmid DNA (Invitrogen). The cells from 3 ml of each overnight culture were collected by centrifugation at 15 000 rpm for 1 min. The supernatant was discarded and the cell pellet suspended in 300 µl of Solution 1 (15 mM Tris-HCl [pH 8]; 10 mM EDTA), after which 300 µl of Solution 2 (0.2 N NaOH; 1% SDS) was added. After incubation at room temperature for 5 min, 300 µl of Solution 3 (3 M KOAc; pH 5.5), was added and the tubes incubated on ice for 10 min. The insoluble material, consisting of proteins and *E. coli* genomic DNA, was removed by centrifugation at 15 000 rpm for 10 min. The bacmid DNA was precipitated from the recovered supernatant by addition of 800 µl of isopropanol and incubation on ice for 10 min. The precipitated bacmid DNA was collected by centrifugation at 15 000 rpm for 15 min, rinsed with 70% ethanol, air-dried at room temperature for 10 min and then suspended in 40 µl of 1 × TE buffer. An aliquot (5 µl) of the extracted bacmid DNA was analyzed by electrophoresis on a 0.5% (w/v) agarose gel at 70 V for 30 min in 1 × TAE buffer.

2.2.10.3 Analysis of recombinant bacmid DNA

Successful transposition of the AHSV-9 NS2 and NS2-eGFP chimeric genes in the bacmid DNA was verified by PCR analysis of the extracted bacmid DNA. For this purpose, the universal pUC/M13 primers, which anneal at sites flanking the mini-*att*Tn7 site, were used. Each of the PCR reaction mixtures (50 µl) contained 50 ng of bacmid DNA, 1 × PCR buffer, 1.5 mM $MgCl_2$, 25 pmol of each of the sense and antisense primer, 2.5 mM of each dNTP and 1 U of SUPERTHERM *Taq* DNA polymerase (Southern Cross Biotechnology). The tubes were placed in a Perkin-Elmer GeneAmp® 2400 thermal cycler. After initial denaturation at 94°C for 3 min, the reactions were subjected to 35 cycles of denaturation at 94°C for 45 s, primer annealing at 52°C for 45 s and elongation at 72°C for 5 min. To test for contamination, reaction mixtures from which the template DNA was omitted were also included. Following PCR, aliquots of the reaction mixtures were analyzed by agarose gel electrophoresis in the presence of an appropriate DNA molecular marker.

2.2.11 Transient expression of AHSV-9 NS2-eGFP in BHK-21 cells

2.2.11.1 Cell culture

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

2.2.11.2 Transfection of BHK-21 cells

BHK-21 cells were transfected with purified recombinant pCMV-Script® plasmid DNA by means of lipofection, using Lipofectamine™ 2000 reagent (Invitrogen) according to the manufacturer's instructions. The cells were seeded in a 24-well tissue culture dish to reach 90% confluency within 24 h of incubation at 37°C in the presence of 5% CO_2. For each transfection, 800 ng of purified recombinant plasmid DNA and 2 µl of the Lipofectamine™ 2000 reagent were separately diluted in 50 µl of MEM medium (lacking serum and antibiotics). Following incubation at room temperature for 5 min, the two solutions were mixed and incubated at room temperature for 20 min. The cell monolayers were prepared for transfection by rinsing the cells three times with 500 µl of antibiotic-free MEM medium, but containing 2.5% (v/v) FBS. After addition of 400 µl of the same medium, the cells were overlayed with the DNA-lipofectamine complexes (100 µl) and the tissue culture dishes incubated at 37°C for 48 h in a CO_2 incubator. As controls, mock-transfected cells and cells transfected with the parental pCMV-Script® vector were included. Following incubation, eGFP and NS2-eGFP protein expression was examined by fluoresence microscopy as described below (Section 2.2.15).

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

2.2.12.1 Cell culture

Spodoptera frugiperda insect cells were propagated at 27°C as monolayers or as suspension cultures in Grace's insect medium (Highveld Biological), supplemented with 10% (v/v) foetal calf serum (FCS) and antibiotics (0.12 mg/ml penicillin G; 0.12 mg/ml streptomycin sulphate; 0.0325 µg/ml Fungizone) (Highveld Biological). The cell density and viability were determined by staining the cells with trypan blue (0.4% [w/v] in 1 × PBS) and by counting on a haemocytometer, as described by Summers and Smith (1987).

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

Recombinant bacmid DNA was transfected into *S. frugiperda* cells with Cellfectin® (Invitrogen) according to the manufacturer's instructions. The cells were seeded in 35-mm-diameter wells (9×10^5 cells/well) and allowed to attach at 27°C for 1 h. For each transfection, a transfection solution was prepared by combining Solution A (50 ng of bacmid DNA in 100 µl of serum- and antibiotic-free Grace's medium) with Solution B (6 µl of Cellfectin® reagent in 100 µl of serum- and antibiotic-free Grace's medium). Following incubation at room temperature for 45 min, 800 µl of Grace's medium (lacking serum and antibiotics) was added. The medium from the attached cells was aspirated and the cells washed twice with 2 ml of Grace's medium (lacking serum and antibiotics), before being overlayed with 1 ml of the diluted lipid-DNA complexes. The transfected cells were incubated at

27°C for 5 h in a moist environment and the transfection mixtures were then removed and replaced with 2 ml of Grace's medium supplemented with 10% (v/v) FCS and antibiotics. The dishes were subsequently incubated at 27°C for 96 h. Following centrifugation of cell suspensions at 5 000 rpm for 5 min, the cell-free supernatants, containing recombinant baculovirus, were 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.

2.2.13 SDS-PAGE analysis of recombinant bacmid-infected *S. frugiperda* cells

2.2.13.1 Preparation of cell lysates

The *S. frugiperda* cells were seeded in 35-mm-diameter wells (1×10^6 cells/well) and either mock-infected or infected with recombinant and wild-type bacmids. Following incubation at 27°C for 72 h, the cells were harvested by pipetting, collected by centrifugation at 3 000 rpm for 5 min and washed in 1 × phosphate-buffered saline (PBS: 137 mM NaCl; 2.7 mM KCl; 4.3 mM Na_2HPO_4; 1.4 mM KH_2PO_4; pH 7.4). The cells were suspended in 40 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% [w/v] glycerol; 10% [v/v] 2-mercaptoethanol; 0.002% [w/v] bromophenol blue) was added to each sample. The samples were denatured by heating to 95°C for 10 min in a boiling water bath.

2.2.13.2 SDS-PAGE analysis

Protein samples were analyzed by electrophoresis in a discontinuous gel system, as described by Laemmli (1970). The 5% stacking gel (5% [w/v] acrylamide; 0.17% [w/v] bis-acrylamide; 125 mM Tris-HCl [pH 6.8]; 0.1% [w/v] SDS) and the 12% separating gel (12% [w/v] acrylamide; 0.34% [w/v] bis-acrylamide; 0.375 mM Tris-HCl [pH 8.8]; 0.1% [w/v] SDS) were each polymerized by addition of 0.08% (w/v) ammonium persulphate and 10 µl of TEMED. Electrophoresis was performed in a Hoefer Mighty Small™ electrophoresis unit at 120 V for 4 h in 1 × TGS buffer (25 mM Tris; 192 mM glycine; 0.1% [w/v] SDS; pH 8.3). Following electrophoresis, the gels were stained for 30 min with 0.125% (w/v) Coomassie brilliant blue (prepared in 50% [v/v] methanol; 10% [w/v] acetic acid), and then destained in a solution containing 25% (v/v) methanol and 10% (v/v) glacial acetic acid until the proteins were visible. The sizes of the resolved proteins were estimated by comparison to reference molecular mass proteins (Pre-stained Protein Molecular Weight Marker; Fermentas).

2.2.14 Western blot analysis

For Western blot analysis, proteins resolved by SDS-PAGE were electrotransferred from the unstained SDS-polyacrylamide gel onto a Hybond™-C^+ nitrocellulose membrane (Amersham Pharmacia Biotech AB). The gel, two sheets of filter paper and nitrocellulose membrane, cut to the same size as the gel,

were equilibrated for 30 min in transfer buffer (25 mM Tris; 186 mM glycine). The proteins were eletroblotted onto the membrane for 2 h at 24 V and 120 mA, using a Mighty Small™ Transphor blotting unit (Hoefer). After transfer, the membrane was rinsed for 5 min in 1 × PBS and non-specific binding sites were blocked by immersing the membrane for 30 min in blocking solution (1% [w/v] fat-free milk powder in 1 × PBS). The membrane was then transferred to 1 × PBS containing an anti-AHSV NS2 polyclonal antibody (Uitenweerde *et al.*, 1995), diluted 1:100 in 1 × PBS, and incubated 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-Aldrich), diluted 1:100 in 1 × PBS, was added to the membrane and then incubated at room temperature for 1 h with agitation. The membrane was washed three times for 5 min each in wash buffer and once for 5 min in 1 × PBS. Calorimetric detection of immunoreactive proteins was performed by immersing the membrane in a freshly prepared enzyme substrate solution (60 mg 4-chloro-1-naphtol in 20 ml ice-cold methanol, mixed with 100 ml of 1 × PBS containing 60 µl of H_2O_2). Once bands became visible, the membrane was rinsed in water and air-dried.

2.2.15 Fluorescence microscopy

BHK-21 cell monolayers transfected with recombinant plasmid DNA and *S. frugiperda* cell monolayers infected with recombinant bacmids were examined for eGFP and NS2-eGFP protein expression on a Zeiss Axiovert 200 fluorescence 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 with a Nikon DMX1200 digital camera.

2.2.16 Transmission electron microscopy (TEM)

Monolayers of *S. frugiperda* cells seeded in 75-cm^2 tissue culture flasks (1 × 10^7 cells/flask) were either mock-infected or infected 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 10 min and rinsed once with 10 ml of 1 × PBS. The cells were fixed at room temperature for 1 h in a solution comprising 2.5% (v/v) glutaraldehyde and 2 mM $MgCl_2$, after which the cells were pelleted by centrifugation at 2 000 rpm for 5 min and the supernatant discarded. The cells were washed three times with 800 µl of 0.075 M NaP buffer (0.15 M $Na_2HPO_4.2H_2O$; 0.15 M $NaH_2PO_4.H_2O$; pH 7.4) and then fixed with 0.5% osmium tetraoxide (OsO_4) at room temperature for 1 h. The cells were pelleted and washed with NaP buffer, as described above, prior to dehydration in 50%, 70%, 90% and 100% acetone. The treatment with 100% acetone was repeated two more times to ensure dehydration. The cells were then infiltrated for 1 h each in different percentages of Quetol (30%, 60% and 100% prepared in acetone). After the second infiltration with pure Quetol for 4 h, the cells were collected by centrifugation, added

to fresh Quetol and polymerized at 60°C for 48 h. Ultra-thin sections were collected on nickel grids, stained with 4% aqueous uranyl acetate, washed in distilled water and counter-stained with Reynolds' lead citrate. The samples were viewed with a Phillips 301 transmission electron microscope.

2.3 RESULTS

2.3.1 Construction of a recombinant pCMV-Script® mammalian expression vector containing an AHSV-9 NS2-eGFP chimeric gene

Towards developing a vector-based RNAi strategy whereby expression of the AHSV-9 NS2 gene could be silenced by shRNAs in mammalian cells, a NS2-eGFP chimeric gene was constructed. The chimeric gene would aid in providing a simple and impartial assay for rapidly screening and assessing the efficiency of gene silencing by different candidate shRNAs at the protein level visually and quantitatively by fluorometry. The chimeric gene was subsequently constructed by making use of a strategy whereby the eGFP reporter gene was fused in-frame to the C-terminus of the NS2 gene, thus ensuring that transcription of the NS2 gene would need to occur in order to obtain fluorescently tagged NS2 protein. For this purpose, the 3'-specific primer used in PCR amplification of the NS2 gene had been designed to amplify a truncated version of the gene, which lacked the TGA stop codon. Moreover, this primer and the 5'-specific primer used in PCR amplification of the eGFP gene were designed in such a manner that they both contained a unique *Eco*RI restriction endonuclease recognition site, which upon restriction enzyme digestion of the respective DNA fragments would allow for their ligation to yield the desired NS2-eGFP chimeric gene.

Towards construction of the NS2-eGFP chimeric gene, the AHSV-9 NS2 and eGFP genes were PCR-amplified, using recombinant pGEX-AHSVNS2 and pGEM-eGFP constructs as template DNA, respectively, together with the appropriate primers (Section 2.2.9). The amplicons, 1.098 kb for NS2 and 720 bp for eGFP, were cloned into pGEM®-T Easy vector DNA to generate pGEM-NS2Trunc and pGEM-eGFP(M), respectively, and then characterized by restriction enzyme digestion and nucleotide sequencing to confirm their integrity (results not shown). Having confirmed that the stop codon and 3' non-coding region was deleted from the NS2 gene, the truncated NS2 gene was recovered from pGEM-NS2Trunc and cloned into the *Bam*HI and *Eco*RI sites of the mammalian expression vector pCMV-Script® to yield pCMV-NS2Trunc. To complete construction of pCMV-NS2-eGFP (Fig. 2.1A), the eGFP gene was recovered from pGEM-eGFP(M) by digestion with both

*Eco*RI and *Xho*I, and cloned into identically prepared pCMV-NS2Trunc. The recombinant plasmid DNA was characterized by restriction enzyme digestion and nucleotide sequencing.

To verify the presence of the NS2-specific insert, plasmid pCMV-NS2Trunc was digested with both *Bam*HI and *Eco*RI and yielded expected DNA fragments corresponding to *ca.* 4.3 kb and 1.098 kb (Fig. 2.1B, lane 6). The recombinant plasmid pCMV-NS2-eGFP was subsequently characterized by digestion with *Bam*HI and *Eco*RI or *Xho*I. Whereas digestion of the recombinant plasmid with both *Eco*RI and *Xho*I yielded two DNA fragments corresponding in size with the pCMV-NS2Trunc vector DNA (5.398 kb) and the cloned eGFP gene (720 bp) (Fig 2.1B, lane 9), digestion with both *Bam*HI and *Eco*RI resulted in excision of the 1.098-kb truncated NS2 gene (Fig. 2.1B, lane 8). These results therefore confirmed that a chimeric NS2-eGFP gene had been constructed successfully in the mammalian expression vector pCMV-Script®.

The cloned NS2-eGFP chimeric gene was subsequently characterized by nucleotide sequencing. Analysis of the obtained nucleotide sequence indicated no nucleotide differences between the NS2-eGFP chimeric gene and the nucleotide sequences of the NS2 and eGFP coding regions, except for deletion of the stop codon from the NS2 gene and the introduction of six nucleotides, specifying the newly introduced *Eco*RI site, to allow for in-frame fusion of the respective genes. Moreover, analysis of the deduced amino acid sequence confirmed that the open reading frame of the NS2-eGFP protein was maintained.

2.3.2 Expression of the AHSV-9 NS2-eGFP chimeric protein in BHK-21 cells

To determine whether the newly constructed NS2-eGFP chimeric gene will be expressed in BHK-21 cells, cell monolayers were mock-transfected and transfected with purified parental pCMV-Script® vector or the recombinant mammalian expression vectors pCMV-NS2-eGFP and pCMV-eGFP. Examination of the cell monolayers by fluorescent microscopy at 48 h post-transfection (Fig. 2.2) indicated that whereas BHK-21 cells transfected with pCMV-NS2-eGFP and pCMV-eGFP fluoresced, no fluorescence was observed in mock-transfected cells or in cells transfected with the parental pCMV-Script® vector. To confirm expression of the NS2-eGFP protein, cell lysates were prepared and analyzed by SDS-PAGE and Western blot analyses. No uniquely expressed protein could be observed in a Coomassie-stained SDS-polyacrylamide gel when compared to mock-transfected cells, while the NS2 antibody yielded

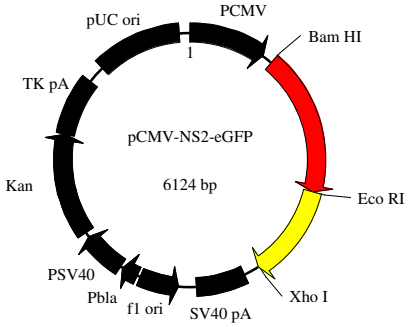

Fig. 2.1A Plasmid map of the recombinant plasmid pCMV-NS2-eGFP, harbouring a chimeric NS2-eGFP gene. The AHSV-9 NS2 gene is indicated in red and the eGFP gene in yellow.

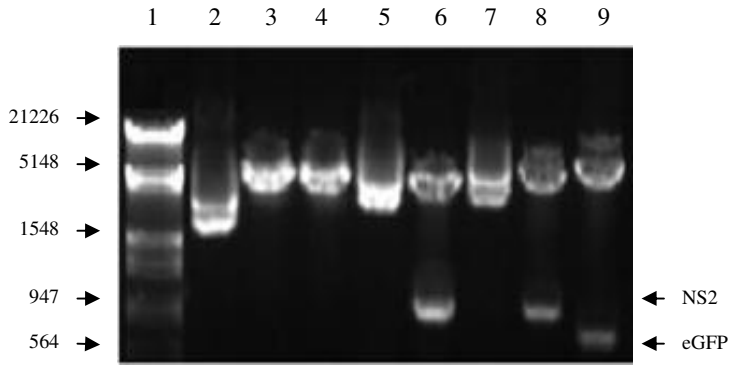

Fig. 2.1B Agarose gel electrophoretic analysis of the recombinant plasmid pCMV-NS2-eGFP. Lane 1, DNA molecular weight marker; lane 2, uncut parental pCMV-Script® vector DNA; lane 3, parental pCMV-Script® vector DNA digested with both *Bam*HI and *Eco*RI; lane 4, parental pCMV-Script® vector DNA digested with both *Eco*RI and *Xho*I; lane 5, uncut pCMV-NS2Trunc plasmid DNA; lane 6, pCMV-NS2Trunc plasmid DNA digested with both *Bam*HI and *Eco*RI; lane 7, uncut pCMV-NS2-eGFP plasmid DNA; lane 8, pCMV-NS2-eGFP plasmid DNA digested with both *Bam*HI and *Eco*RI; lane 9, pCMV-NS2-eGFP plasmid DNA digested with both *Eco*RI and *Xho*I. The sizes of the DNA molecular weight marker, phage λ DNA digested with *Eco*RI and *Hind*III, are indicated to the left of the figure in base pairs.

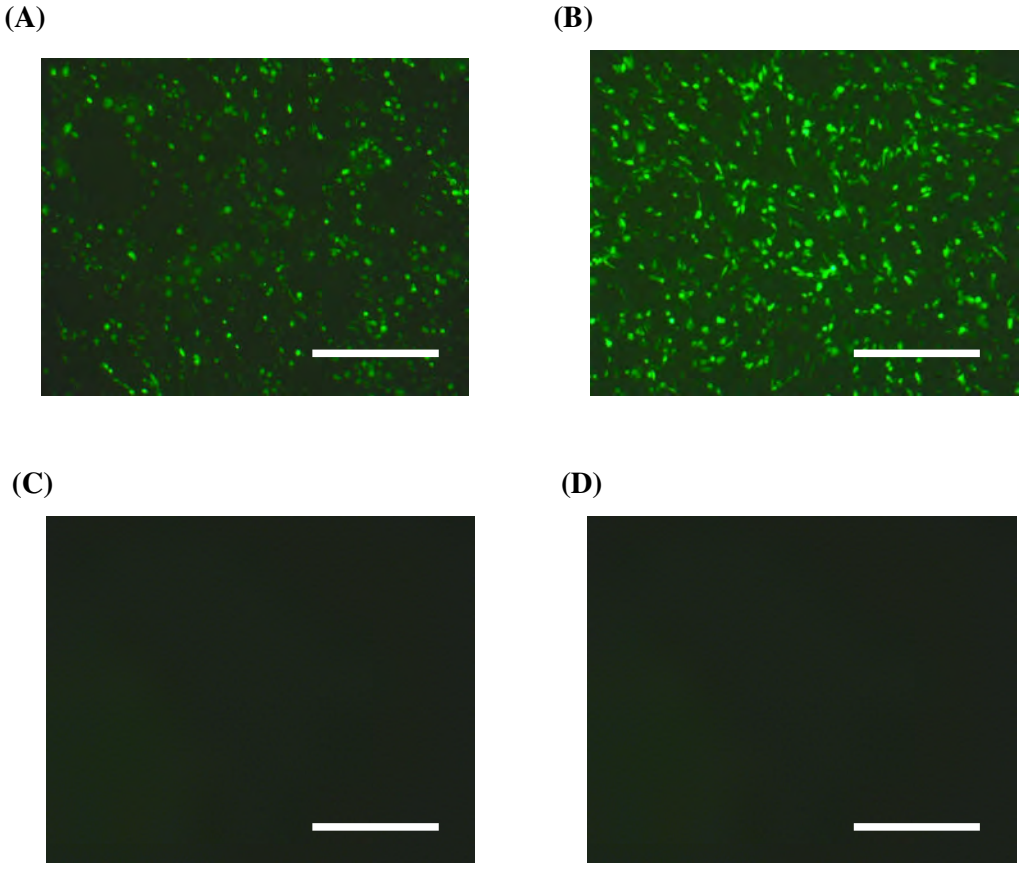

Fig. 2.2 Fluorescence microscopy of NS2-eGFP and eGFP gene expression in BHK-21 cells. BHK-21 cells transfected with pCMV-NS2-eGFP (A) and pCMV-eGFP (B) were analyzed for protein expression 48 h post-transfection, using a Zeiss Axiovert fluorescent microscope. Mock-transfected cells (C) and cells transfected with pCMV-Script® vector DNA (D), were included as controls. Bar = 500 µm.

several non-specific immunoreactive bands in Western blot analysis (results not shown). These results are most likely due to the low levels of transient gene expression, which is often the case when using mammalian expression systems (Yin *et al.*, 2007). Consequently, it was decided to express the NS2-eGFP chimeric gene in *S. frugiperda* insect cells by means of a recombinant bacmid in order to characterize the chimeric protein.

2.3.3 Construction of recombinant pFastBac1™ donor plasmids containing the NS2 and NS2-eGFP chimeric genes

To enable cloning of the AHSV-9 NS2 gene into the bacmid donor plasmid pFastBac1™, both the pCMV-NS2 and pFastBac1™ plasmid DNAs were digested with *Bam*HI. The excised full-length NS2 gene and digested vector DNA were purified from an agarose gel and ligated overnight. Similarly, the NS2-eGFP chimeric gene was excised from recombinant plasmid pCMV-NS2-eGFP by digestion with both *Bam*HI and *Xho*I and then ligated into identically prepared pFastBac1™ vector DNA. Following transformation of competent *E. coli* DH5α cells, plasmid DNA isolated from randomly selected transformants was characterized by agarose gel electrophoresis. Plasmid DNA migrating slower than the parental pFastbac1™ vector DNA were selected and characterized by restriction enzyme digestion.

Digestion of recombinant bacmid donor plasmid pFB-NS2 (Fig. 2.3A) with *Bam*HI resulted in the excision of a 1.116-kb DNA fragment (Fig. 2.3B, lane 6), indicating that the full-length AHSV-9 NS2 gene had been cloned successfully. To verify the correct transcriptional orientation of the cloned NS2 gene, the recombinant bacmid donor plasmid was characterized by restriction enzyme mapping with *Hind*III, which recognizes a site within the multiple cloning site of the vector and cuts asymmetrically within the NS2 gene, *i.e.* 337 bp from the 5' end. Digestion of pFB-NS2 with *Hind*III excised an expected DNA fragment of 779 bp (Fig. 2.3B, lane 7).

To verify that the NS2-eGFP chimeric gene had been cloned successfully, the recombinant bacmid donor plasmid pFB-NS2-eGFP was characterized by digestion with different restriction enzymes. Whereas digestion of the recombinant plasmid DNA with both *Bam*HI and *Eco*RI excised a DNA fragment corresponding in size with the truncated NS2 gene (1.098 kb), digestion of the plasmid DNA with both *Eco*RI and *Xho*I excised a DNA fragment corresponding in size with the eGFP gene (720 bp) (Fig. 2.3C, lanes 6 and 7, respectively).

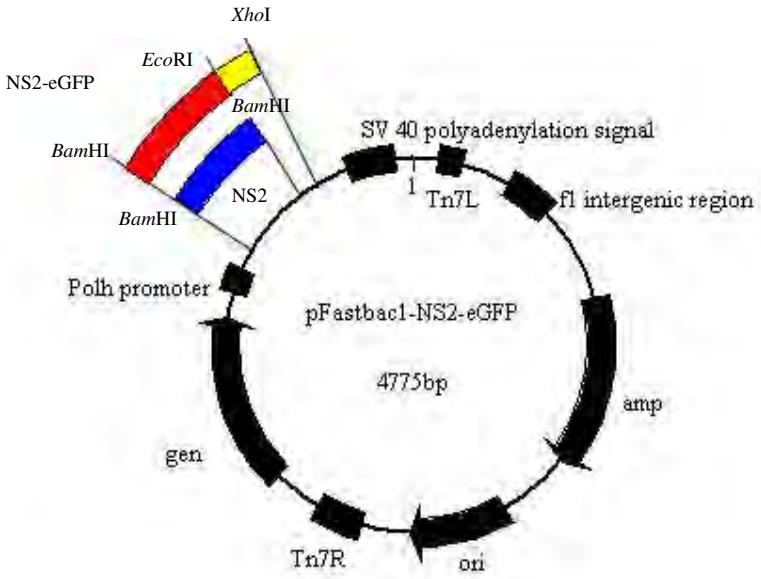

Fig. 2.3A Plasmid map of the recombinant plasmids pFB-NS2 and pFB-NS2-eGFP harbouring a full-length NS2 gene (1.116 kb) and a chimeric NS2-eGFP gene (1.818 kb), respectively.

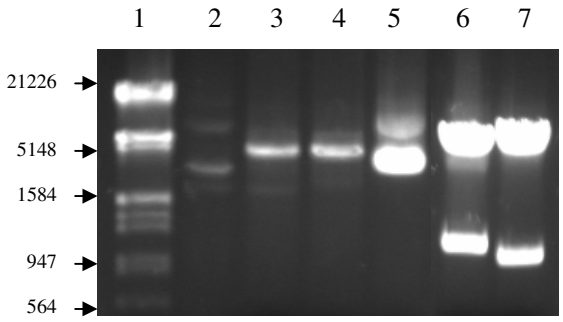

Fig. 2.3B Agarose gel electrophoretic analysis of the recombinant donor plasmid pFB-NS2. Lane 1, DNA molecular weight marker; lane 2, uncut parental pFastBac1™ vector DNA; lane 3, parental pFastBac1™ vector DNA digested with *Bam*HI; lane 4, parental pFastBac1™ vector DNA digested with *Hind*III; lane 5, uncut pFB-NS2 plasmid DNA; lane 6, pFB-NS2 plasmid DNA digested with *Bam*HI; lane 7, pFB-NS2 plasmid DNA digested with *Hind*III. The sizes of the DNA molecular weight marker, phage λ DNA digested with *Eco*RI and *Hind*III, are indicated to the left of the figure in base pairs.

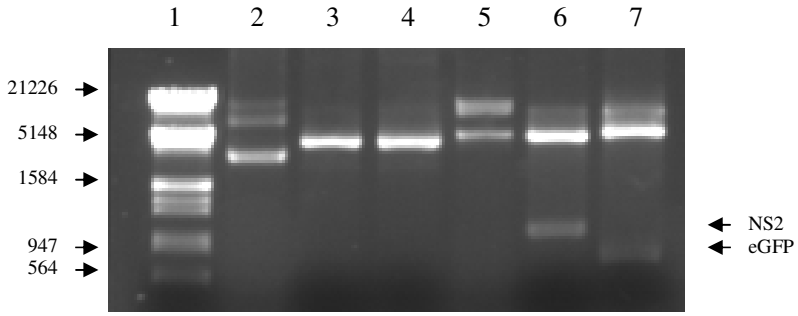

Fig. 2.3C Agarose gel electrophoretic analysis of the recombinant donor plasmid pFB-NS2-eGFP. Lane 1, DNA molecular weight marker; lane 2, uncut parental pFastBac1™ vector DNA; lane 3, parental pFastBac1™ vector DNA digested with both *Bam*HI and *Eco*RI; lane 4, parental pFastBac1™ vector DNA digested with both *Eco*RI and *Xho*I; lane 5, uncut pFB-NS2-eGFP; lane 6, pFB-NS2-eGFP plasmid DNA digested with both *Bam*HI and *Eco*RI; lane 7, pFB-NS2-eGFP plasmid DNA digested with both *Eco*RI and *Xho*I. The sizes of the DNA molecular weight marker, phage λ DNA digested with *Eco*RI and *Hind*III, are indicated to the left of the figure in base pairs.

2.3.4 Construction of recombinant bacmids

The bacmid DNA propagates 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 site-specific transposition of the recombinant donor plasmid, the mini-Tn7 cassette is inserted from the donor plasmid into the mini-*att*Tn7 attachment site on the bacmid DNA, thereby disrupting the expression of the *lacZα* peptide. Thus, colonies containing the recombinant bacmid display a white colony-phenotype and can be readily distinguished from the blue colonies that harbour the unaltered bacmid DNA. The transposase required for the transposition is encoded by the pMON7124 helper plasmid, which also confers resistance to tetracycline (Luckow *et al.*, 1993).

Recombinant bacmid DNA was engineered by transforming competent *E. coli* DH10Bac™ cells with the recombinant pFB-NS2 and pFB-NS2-eGFP donor plasmids, and selecting for recombinant bacmids by plating the transformed cells onto a selective medium. The high-molecular-weight recombinant bacmid DNA was subsequently extracted from selected transformants and used as template DNA in PCR assays to confirm successful transposition of the respective genes into the bacmid DNA. For this purpose, the universal pUC/M13 forward and reverse primers were used, which anneal to sequences flanking the mini-*att*Tn7 attachment site within the *lacZα* gene of the bacmid DNA (Fig. 2.4A).

By making use of recombinant bacmid DNA transposed with either the pFB-NS2 or the pFB-NS2-eGFP donor plasmids as templates for PCR, bands of *ca.* 3.369 kb (Fig. 2.4B, lanes 3-5) and 4.071 kb (Fig. 2.4C, lanes 3-5) were obtained, respectively. These corresponded with the size of the NS2 gene (1.116 kb) or NS2-eGFP chimeric gene (1.818 kb), together with the size of the transposed mini-Tn7 cassette (1.980 kb) and bacmid DNA flanking the mini-*att*Tn7 site (273 bp). As expected, a 273-bp amplicon was observed in PCR reactions in which wild-type bacmid DNA was used as template (Figs. 2.4B and 2.4C, lanes 2), and no amplification products were observed in control reactions from which template DNA was omitted. Recombinant bacmids were selected, designated Bac-NS2 and Bac-NS2-eGFP, respectively, and used in all subsequent experiments.

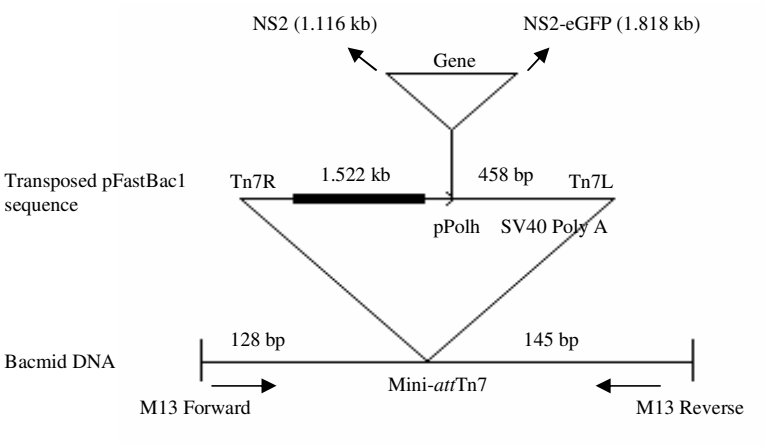

Fig. 2.4A Diagrammatic representation of the bacmid transposition region, indicating the annealing positions of the universal pUC/M13 forward and reverse primers.

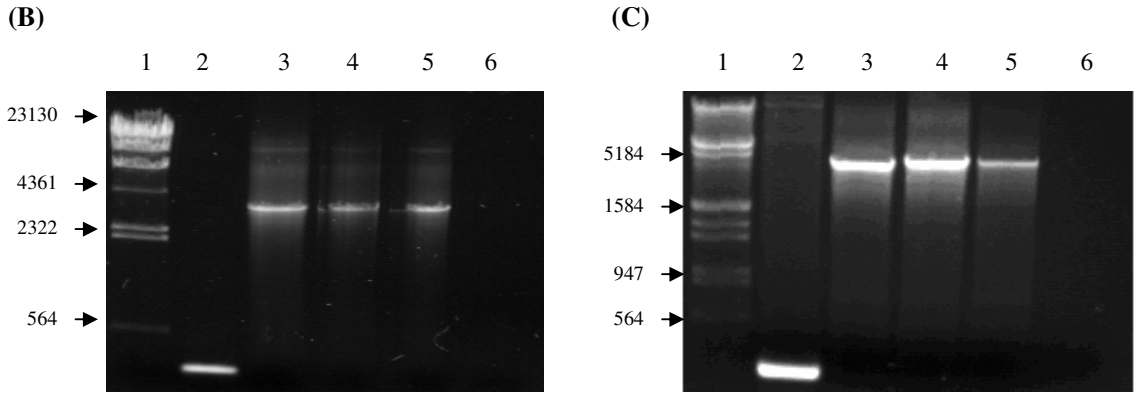

Fig. 2.4B,C Agarose gel electrophoretic analysis of recombinant Bac-NS2 DNA (B) and Bac-NS2-eGFP DNA (C), following PCR analysis with the universal pUC/M13 forward and reverse primers. In both figures: Lane 1, DNA molecular weight marker; lane 2, amplicon obtained using wild-type bacmid DNA as template; lanes 3 to 5, amplicons obtained using recombinant bacmid DNA as template; lane 6, negative control PCR reaction mixture lacking template DNA. The sizes of the DNA molecular weight marker, phage λ DNA digested with *Hind*III (A) and with both *Eco*RI and *Hind*III (B), are indicated to the left of the figure in base pairs.

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

Wild-type and recombinant bacmid DNA were transfected into *S. frugiperda* cells and the progeny viruses were collected four days post-transfection. To determine whether the AHSV-9 NS2 protein and NS2-eGFP chimeric protein were synthesized in recombinant bacmid-infected cells, monolayers of *S. frugiperda* cells were mock-infected and infected with Bac-NS2, Bac-NS2-eGFP and wild-type bacmid. Following incubation at 27°C for 72 h, the monolayers were examined by fluorescence microscopy, after which the cells were harvested and the whole-cell lysates analyzed by SDS-PAGE and Western blot analyses.

Examination of the respective cell monolayers by fluorescence microscopy (Fig. 2.5) indicated that cells infected with the Bac-NS2-eGFP bacmid recombinant fluoresced brightly and, as expected, no fluorescence was observed in control mock-infected cells or in cells infected with the wild-type bacmid or Bac-NS2 bacmid recombinant. Analysis of the Coomassie blue-stained gel (Fig. 2.6A) indicated the presence of a unique protein in the lysates prepared from cells infected with Bac-NS2 (Fig. 2.6A, lanes 4 and 5) and Bac-NS2-eGFP (Fig. 2.6A, lane 6). The estimated molecular mass of the respective proteins was in agreement with the predicted molecular mass of NS2 (41 kDa) and the NS2-eGFP chimeric protein (68 kDa). No similar proteins were observed in the lysates prepared from either mock-infected or wild-type bacmid-infected cells (Fig. 2.6A, lanes 2 and 3, respectively). Subsequent Western blot analysis of the cell lysates indicated that the uniquely expressed proteins reacted specifically with the anti-AHSV-9 NS2 antibody (Fig. 2.6B), thus confirming that the proteins being expressed were indeed the AHSV-9 NS2 and NS2-eGFP proteins.

2.3.6 Transmission electron microscopy of bacmid-infected *S. frugiperda* cells

The NS2 protein has been shown to be the main component of virus inclusion bodies (VIBs) that appear in the cytoplasm of bluetongue virus (BTV)-infected mammalian cells (Hyatt and Eaton, 1988). It has also been reported that baculovirus-expressed NS2 of BTV and AHSV can form similar inclusion bodies in the absence of any other viral proteins (Brookes *et al.*, 1993; Uitenweerde *et al.*, 1995). To determine whether addition of the C-terminal eGFP reporter may influence the ability of NS2 to form inclusion bodies, monolayers of *S. frugiperda* cells were infected with the respective recombinant bacmids and processed for transmission electron microscopy. Ultra-thin sections were collected on nickel grids, contrasted and then viewed with a Phillips transmission electron microscope.

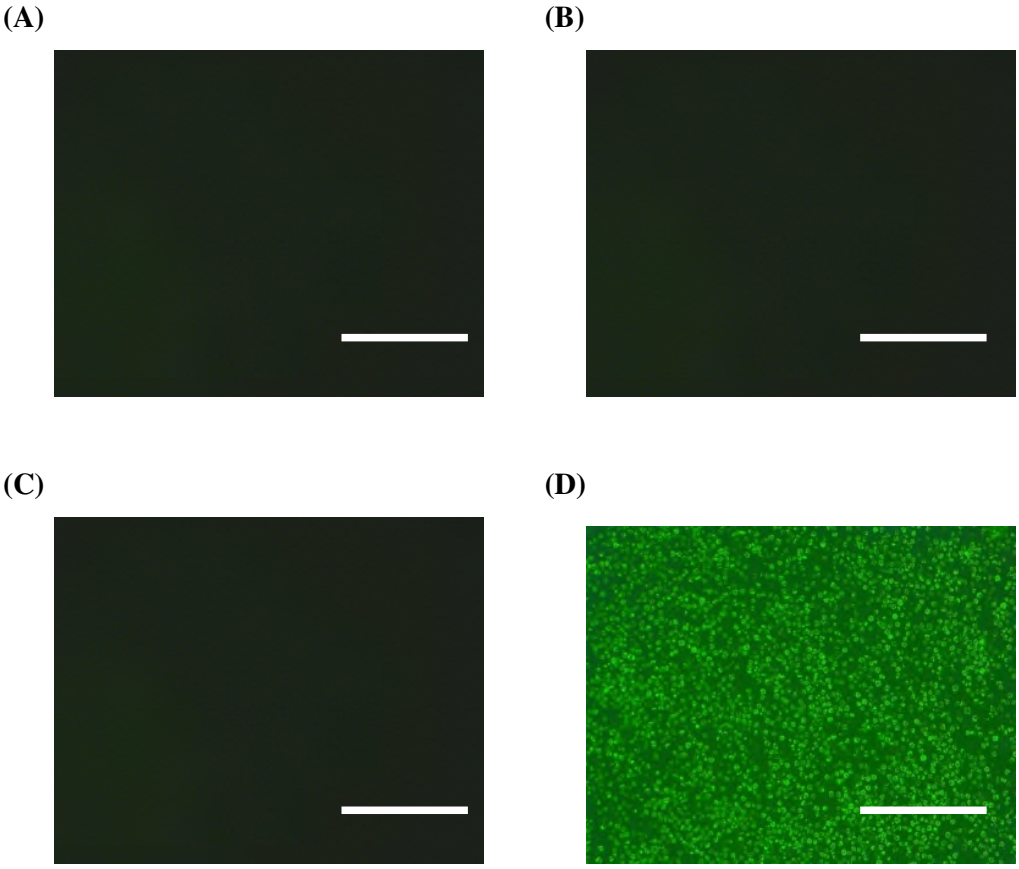

Fig. 2.5 Fluorescence microscopy of recombinant bacmid-infected *S. frugiperda* cells. *S. frugiperda* cells were mock-infected (A) and infected with the wild-type bacmid (B) or recombinant bacmids Bac-NS2 (C) and Bac-NS2-eGFP (D), and analyzed for protein expression at 72 h post-infection using a Zeiss Axiovert fluorescent microscope. Bar = 500 µm.

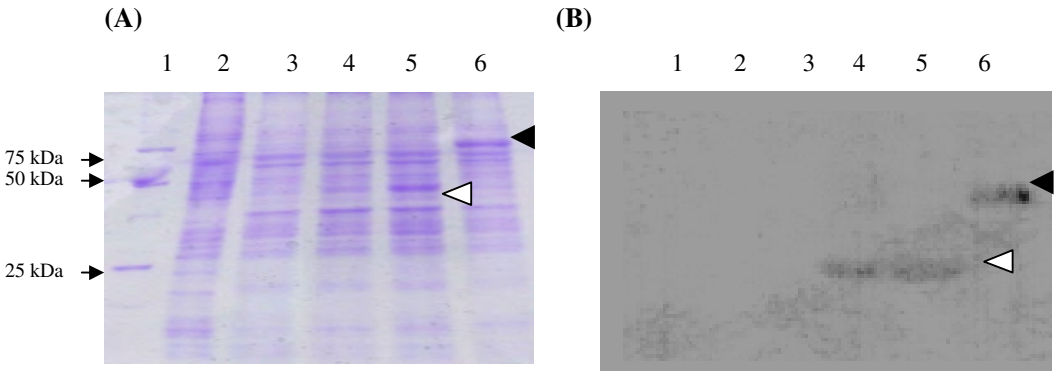

Fig. 2.6 SDS-PAGE and Western blot analyses of whole-cell lysates prepared from recombinant bacmid-infected *S. frugiperda* cells. The cell lysates were resolved by SDS-PAGE (A) and a duplicate, unstained gel was electroblotted onto a nitrocellulose membrane and subjected to Western blot analysis with an anti-AHSV-9 NS2 antibody (B). In both figures: Lanes 1, Protein molecular weight marker; lanes 2, mock-infected cells; lanes 3, wild-type bacmid-infected cells; lanes 4 and 5, Bac-NS2 infected cells; lanes 6, Bac-NS2-eGFP infected cells. The sizes of the protein molecular weight marker are indicated to the left of the figure. The NS2 and NS2-eGFP proteins are indicated by open and closed arrows, respectively.

The results from the electron microscope analysis (Fig. 2.7) indicated that granular electron-dense structures were present in the cytoplasm of *S. frugiperda* cells infected with Bac-NS2 and Bac-NS2-eGFP, respectively. Notably, the inclusion bodies formed by the NS2-eGFP protein appeared to be indistinguishable from those formed by the AHSV-9 NS2 protein. No similar structures were, however, observed in control mock-infected or in wild-type bacmid-infected cells. These results therefore suggested that the addition of the C-terminal eGFP tag did not adversely affect the ability of the AHSV-9 NS2 protein to form inclusion bodies.

2.4 DISCUSSION

The goal with most RNAi protocols is to effectively reduce protein levels in cells and to study the functional consequences of their removal. The key challenge for achieving effective gene silencing is primarily dependant on the effectiveness and specificity of the RNAi effector molecule. However, only a limited number of si/shRNAs is capable of inducing highly effective and sequence-specific gene silencing (Reynolds *et al.*, 2004; Taxman *et al.*, 2006). In addition, the efficiency of si/shRNA-induced gene silencing can only be experimentally measured based on inhibition of the target gene expression. Therefore, it is important to use a robust and comparative validating system for determining the efficiency of different si/shRNA. In this part of the investigation, an NS2-eGFP chimeric gene was thus constructed and characterized with a view towards using it in a quantitative reporter-based shRNA validation system.

Although various different target gene-reporter based si/shRNA validation systems have been described, they typically rely on the use of luciferase, epitope tags or eGFP as the reporter gene (Yokota *et al.*, 2003; Wu *et al.*, 2004; Liu *et al.*, 2006; Lantermann *et al.*, 2007). In contrast to the other reporters, which require an enzyme substrate or antibodies to detect the reporter protein, the fluorescence from eGFP does not require additional co-factors, substrates or other gene products (Chalfie *et al.*, 1994). Moreover, the eGFP reporter protein contains chromatophore mutations that make the protein 35 times brighter than wild-type GFP and has been codon-optimized for high expression in mammalian cells (Chiu *et al.*, 1996; Cormack *et al.*, 1996). Like wild-type GFP, eGFP fluorescence is stable, species-independent and can be monitored by fluorescence microscopy, flow cytometry and fluorometry (Zhang *et al.*, 1996; Stassen *et al.*, 2007). Consequently, eGFP was specifically chosen as reporter gene

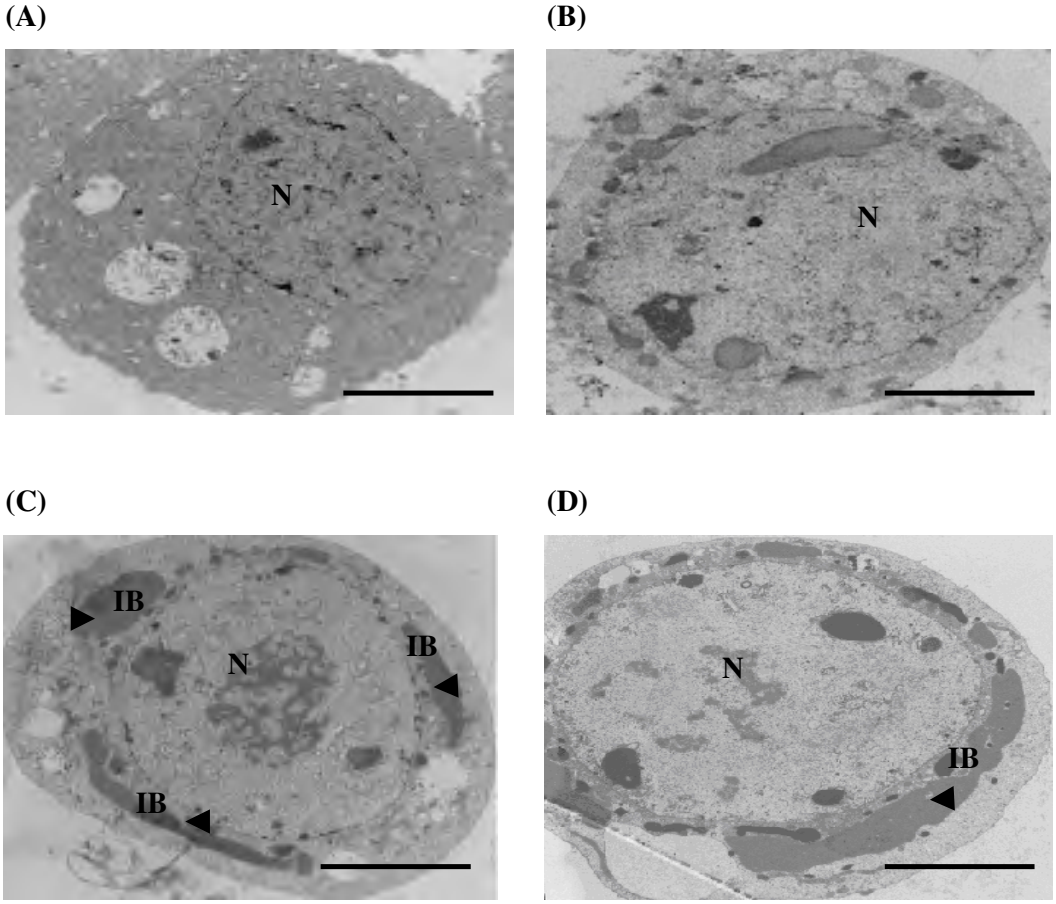

Fig. 2.7 Representative transmission electron micrographs of thin sections of *S. frugiperda* cells infected with different recombinant bacmids. The cells were either mock-infected (A) or infected with the wild-type bacmid (B), and recombinant bacmids Bac-NS2 (C) or Bac-NS2-eGFP (D). The inclusion bodies are indicated by arrows. Abbreviations: N, nucleus; IB, inclusion body. Bar = 10 µm.

in this study since the ability of the NS2-directed shRNAs to silence its target gene can be analyzed readily by a visual decrease in the number of green fluorescent cells when viewed under a fluorescent microscope and quantitatively by fluorometry (Stassen *et al.*, 2007).

A chimeric AHSV-9 NS2-eGFP gene was initially constructed in the pCMV-Script® mammalian expression vector and expression of the NS2-eGFP protein in transfected BHK-21 cells was evidenced by fluorescing cells. However, expression of the NS2-eGFP protein could not be confirmed by Western blot analyses, probably as a consequence of the low levels of transient gene expression, which appears to often be the case when using mammalian expression systems (Yin *et al.*, 2007). To characterize the NS2-eGFP chimeric protein, recombinant bacmids were subsequently generated that harboured a cloned copy of the NS2 and NS2-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). Using this promoter, the expression of foreign proteins can represent up to 50% of the total proteins of the infected host cell. SDS-PAGE analysis of cell lysates prepared from the recombinant bacmid-infected *S. frugiperda* cells indicated the presence of a 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 Western blot analysis and the NS2 antibody reacted specifically with the AHSV NS2 (41 kDa) and chimeric NS2-eGFP (68 kDa) proteins. In addition, examination of thin sections of the recombinant bacmid-infected cells by electron microscopy indicated that the inclusion bodies formed by the NS2 and NS2-eGFP proteins were morphologically indistinguishable from each other.

In addition to its use in a quantitative reporter-based shRNA validation system, the availability of a NS2-eGFP chimeric gene may also be used to investigate the spatial organization of NS2 gene expression in mammalian cells at the single cell level, using sensitive imaging cameras and fluorescence microscopy. Indeed, eGFP has been extensively and selectively used as a fusion partner for different viral proteins to monitor their intracellular localization, trafficking and fates (Slack *et al.*, 2001; Ward and Moss, 2001; Husain and Moss, 2003; Finke *et al.*, 2004; Le *et al.*, 2006; Ebihara *et al.*, 2007). In the case of bluetongue virus (BTV), a related orbivirus, a VP2-eGFP chimeric protein was used not only to assess the intracellular distribution of VP2, but also to demonstrate that VP2 is found associated with vimentin and that this interaction may contribute to virus egress (Bhattacharya

et al., 2007). Using a similar approach, Kar *et al.* (2005) constructed an eGFP-VP3 chimeric protein to examine VP3 localization and degradation in the presence and absence of other BTV proteins. It was furthermore reported that eGFP had no effect on the interaction between the chimeric VP3 protein and VP7, and that eGFP-VP3 assembled correctly into subcores. The latter is similar to the results obtained in this study in so far as the eGFP reporter protein did not affect the ability of NS2-eGFP to form inclusion bodies in the cytoplasm of recombinant bacmid-infected insect cells.

In conclusion, the results obtained in this part of the investigation indicated that an AHSV-9 NS2-eGFP chimeric gene was constructed successfully and that the chimeric NS2 protein could be expressed in both insect cells and mammalian cells. The mammalian expression construct pCMV-NS2-eGFP was subsequently used as a reporter vector to screen different NS2-directed shRNAs for their ability to silence AHSV-9 NS2 gene expression, the details of which are presented in the following Chapter.

CHAPTER THREE

INHIBITION OF AHSV-9 NS2 EXPRESSION BY VECTOR-EXPRESSED SHORT HAIRPIN RNA (shRNA) IN BHK-21 CELLS

3.1 INTRODUCTION

African horse sickness virus (AHSV) is a member of the *Orbivirus* genus within the *Reoviridae* family and has a 10-segment double-stranded (ds)RNA genome enclosed within a double capsid (Bremer, 1976; Bremer *et al.*, 1990; Grubman and Lewis, 1992). The segments code for seven capsid and viral core proteins (VP1-VP7). The remaining three segments encode non-structural proteins (NS1, NS2 and NS3/NS3A) that are produced in the infected host cell at different stages of the replication cycle and are presumed to be involved in the various stages of viral morphogenesis (Roy *et al.*, 1994b; Roy, 2001; Huismans *et al.*, 2004; Stuart and Grimes, 2006).

AHSV RNA segment 8 encodes NS2 (41 kDa), which is the only viral protein reported to be phosphorylated (Devaney *et al.*, 1988), and has a high affinity for single-stranded (ss)RNA (Uitenweerde *et al.*, 1995). It has subsequently been reported that NS2 displays preferential and specific binding to viral RNA (Theron and Nel, 1997; Lymperopoulos *et al.*, 2003; Markotter *et al.*, 2004; Lymperopoulos *et al.*, 2006). The NS2 protein is thus of particular interest because of its possible involvement in genome recognition (preference for viral ssRNA versus cellular RNA), which is known to be an early essential step in the assembly process of viruses. Moreover, the NS2 protein is synthesized in large amounts throughout the replication cycle and accumulates in large electron-dense structures, termed virus inclusion bodies (VIBs) (Thomas *et al.*, 1990; Brookes *et al.*, 1993; Uitenweerde *et al.*, 1995). The presence of core and morphogenesis intermediates in and around the periphery of VIBs has led to the proposal that virus replication and assembly occur at these sites (Hyatt and Eaton, 1988; Hyatt *et al.*, 1992; Modrof *et al.*, 2005; Kar *et al.*, 2007).

RNA interference (RNAi) is a process of sequence-specific, post-transcriptional gene silencing that is mediated by short dsRNA (Fire *et al.*, 1998; Tuschl *et al.*, 1999; Elbashir *et al.*, 2001b). However, in mammalian cells, the introduction of dsRNA longer than 30 bp provokes a strong cytotoxic response that leads to the non-specific degradation of RNA transcripts and a general shutdown of host cell protein translation (Manche *et al.*, 1992; Williams; 1997; Stark *et al.*, 1998). This problem has been overcome by using chemically synthesized small interfering RNA (siRNA) that is typically 21 to 23 nt in length. The synthetic siRNA is long enough to mediate gene-specific silencing, but is short enough to evade the adverse effects of dsRNA longer that 30 bp (Elbashir *et al.,* 2001a; Elbashir *et al.,*

2001b; Caplen *et al.*, 2001). Since the first publications to report that RNAi can be used to suppress viral gene expression in mammalian cells (Bitko and Barik, 2001; Gitlin *et al.*, 2002), RNAi has become a powerful tool not only for the analysis of viral gene function (Déctor *et al.*, 2002; McManus and Sharpe, 2002; Campagna *et al.*, 2005; Wirblich *et al.*, 2006), but also as a new therapeutic approach for controlling virus infections (Haasnoot *et al.*, 2003; Tan and Yin, 2004; Colbère-Garapin *et al.*, 2005; Stram and Kuzntzova, 2006).

Although significant gene silencing can be achieved with transfection of chemically synthesized siRNA into mammalian cells, there are several limitations to this approach, including the efficiency of delivery of the siRNA duplex into certain cell lines, the lack of long-term gene silencing and the relatively high cost of chemical synthesis (Elbashir *et al.*, 2002; Agrawal *et al.*, 2003). Consequently, numerous publications have reported the development of plasmid DNA-based vectors (Brummelkamp *et al.*, 2002a; Paddison *et al.*, 2002; Sui *et al.*, 2002; Gupta *et al.*, 2004; Wu *et al.*, 2005) and viral vectors (Brummelkamp *et al.*, 2002b; Xia *et al.*, 2002; Rubinson *et al.*, 2003; Qin *et al.*, 2003; Liu *et al.*, 2004b; Grimm *et al.*, 2005) that are capable of generating siRNA-like molecules within cells. After transfection into cells the vector expresses short hairpin RNA (shRNA), which makes a tight hairpin turn, and can be digested by Dicer to siRNA that subsequently guides the cleavage of complementary mRNA. Added advantages of these vector-based RNAi systems are that they are economical to use and have the ability to mediate persistent gene silencing (Amarzguioui *et al.*, 2005; Sandy *et al.*, 2005).

Towards the long-term goal of investigating the *in vivo* functional role of NS2 during virus replication and morphogenesis, the principal aim of this part of the study was to develop a plasmid DNA vector-based RNAi assay whereby expression of the AHSV-9 NS2 gene could be silenced in BHK-21 cells. For this purpose, recombinant pSUPER shRNA delivery vectors were constructed, each targeting a distinct 19-nt sequence in the NS2 gene. Their efficiency to inhibit expression of a previously constructed chimeric NS2-eGFP reporter gene (Chapter 2), as well as NS2 gene expression in AHSV-9 infected BHK-21 cells was subsequently evaluated. Based on reports demonstrating that retroviral vectors can be used to integrate shRNA expression cassettes into the genome of mammalian cells (Brummelkamp *et al.*, 2002b; Liu *et al.*, 2004b; Musiyenko *et al.*, 2007), it was also investigated whether the pSUPER.Retro.Puro retroviral vector could be used to generate a shRNA-expressing BHK-21 cell line in which the AHSV-9 NS2 gene is stably suppressed.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial strains and plasmids

The *Escherichia 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) at 37°C with shaking at 200 rpm, and maintained at 4°C on LB agar or at -70°C as glycerol cultures. For plasmid DNA selection and maintenance in *E. coli*, the culture medium was supplemented with 50 µg/ml of ampicillin (Sigma-Aldrich). The recombinant mammalian expression vector pCMV-NS2-eGFP had been constructed previously (Chapter 2), while the shRNA delivery vectors pSUPER and pSUPER.Retro.Puro were obtained from OligoEngine.

3.2.2 Oligonucleotides for shRNA construction

Oligonucleotides used to produce shRNAs that targeted three distinct regions in the NS2 gene were designed based on the AHSV-9 NS2 sequence (GenBank Acc. no. M69090) and by making use of different siRNA design tools, *i.e.* Ambion siRNA Target Finder (available at http://www.ambion.com), Qiagen siRNA Design Tool (available at http://www.qiagen.com) and Dharmacon siRNA Design Tool (available at http://www.dharmacon.com). The oligonucleotide sequences were subsequently compared to 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 target regions was evaluated by RNA secondary structure analyses of the NS2 and chimeric NS2-eGFP mRNA with MFOLD v.3.1 hybridization and folding software (Zuker, 2003) (Figs. 3.1 and 3.2). The sequence of each forward oligonucleotide included the 19-nt target sequence in both sense and antisense orientation, separated by a 9-nt loop sequence (5'-TTCAAGAGA-3'). The 5' end contained a 4-nt overhang to create a *Bgl*II restriction site (GATC), while the 3' end contained a RNA polymerase H1 termination sequence (five thymidine residues). The complementary reverse oligonucleotide contained a 4-nt overhang at the 5' end to create a *Hind*III restriction site (AGCT), but no 4-nt *Bgl*II restriction site at the 3' end. A control non-silencing shRNA (shRNA-Uneg) was designed similarly, using a sequence that reportedly lacks homology to all known viral and cellular genes (Qiagen). The oligonucleotides used for shRNA construction are shown in Table 3.1 and were obtained from Qiagen.

3.2.3 Plasmid DNA construction

All molecular cloning techniques used in the construction of recombinant pSUPER and pSUPER.Retro.Puro shRNA delivery vectors were performed according to the manufacturer's protocol, and according to the procedures described in Chapter 2 (Sections 2.2.2 through 2.2.8). All plasmid constructs were confirmed by restriction endonuclease digestion and by nucleotide sequencing.

Table 3.1 Oligonucleotides used for shRNA construction

Oligonucleotide	Nucleotide sequence	NS2 coding region targeted
UnegF	5' - GATCCCC**AATTCTCCGAACGTGTCACGT***TTCAAGAGA***ACGTGACACGTTCGGAGAATT**TTTTTGGAAA - 3'	None
UnegR	5' - AGCTTTTCCAAAAA**AATTCTCCGAACGTGTCACGT***TCTCTTGAA***ACGTGACACGTTCGGAGAATT**GGG - 3'	None
211F	5' - GATCCCC**GTATTCAAGATGGGACGGATG***TTCAAGAGA***CATCCGTCCCATCTTGAATAC**TTTTTGGAAA - 3'	211-232
211R	5' - AGCTTTTCCAAAAA**GTATTCAAGATGGGACGGATG***TCTCTTGAA***CATCCGTCCCATCTTGAATAC**GGG - 3'	211-232
377F	5' - GATCCCC**AAGCGGTGCGGTTCCACCTTA***TTCAAGAGA***TAAGGTGGAACCGCACCGCTT**TTTTTGGAAA - 3'	377-398
377R	5' - AGCTTTTCCAAAAA**AAGCGGTGCGGTTCCACCTTA***TCTCTTGAA***TAAGGTGGAACCGCACCGCTT**GGG - 3'	377-398
958F	5' - GATCCCC**AATGGGAGAATGTTCCGATCT***TTCAAGAGA***AGATCGGAACATTCTCCCATT**TTTTTGGAAA - 3'	958-979
958R	5' - AGCTTTTCCAAAAA**AATGGGAGAATGTTCCGATCT***TCTCTTGAA***AGATCGGAACATTCTCCCATT**GGG - 3'	958-979

- Nucleotides indicated in bold represent the sense and antisense shRNA target sequences
- Nucleotides indicated in italics represent the loop sequence required for hairpin formation of transcripts
- Nucleotides indicated in blue and red represent *Bgl*II and *Hind*III restriction enzyme overhangs, respectively

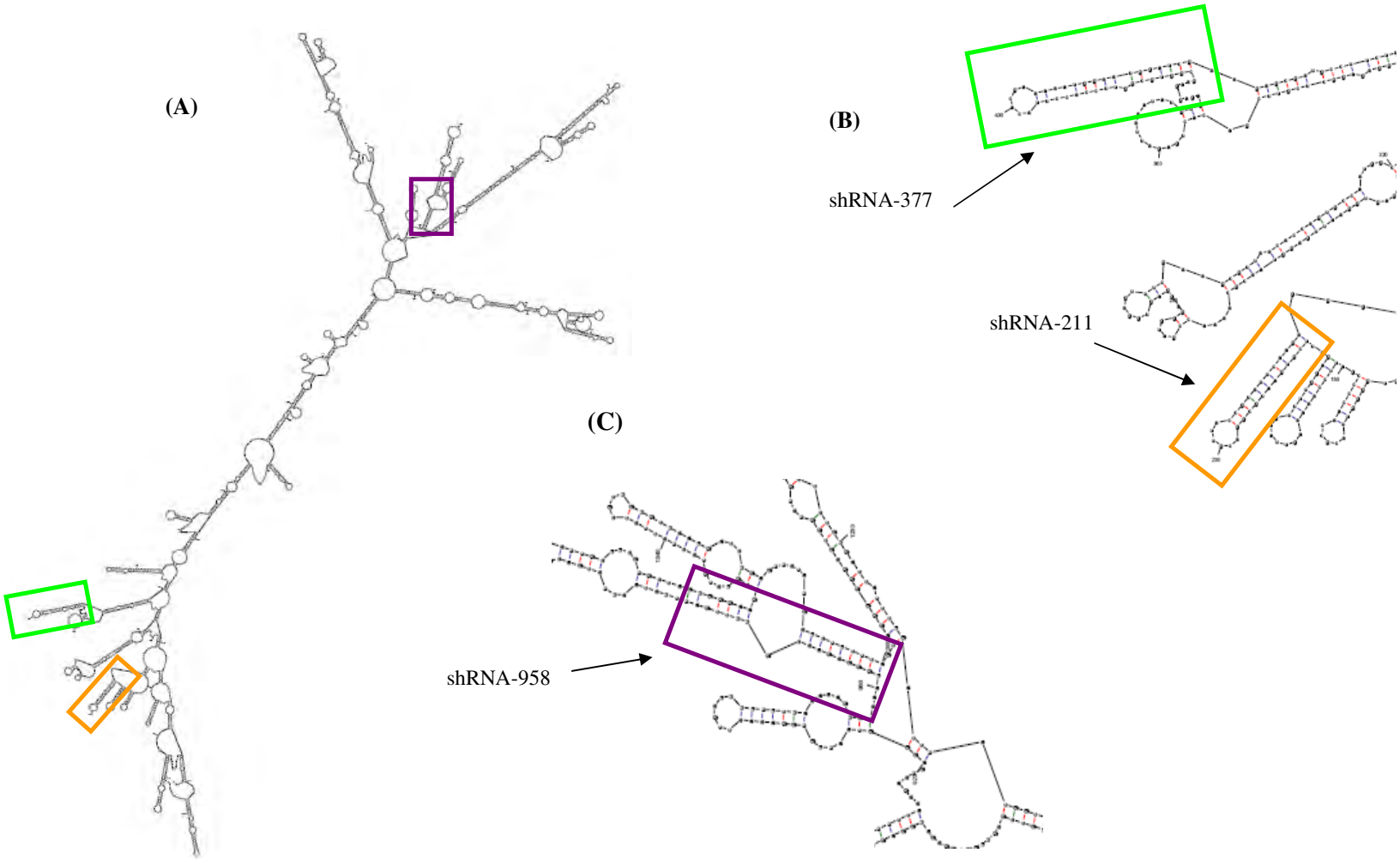

Fig. 3.1 Local secondary structure of the chimeric AHSV-9 NS2-eGFP mRNA (A) and close-up views (B and C) of the shRNA target sites, as predicted by the MFOLD software program (Zuker, 2003). The regions targeted by the NS2-directed shRNAs are boxed in orange (shRNA-211), green (shRNA-377) and purple (shRNA-958).

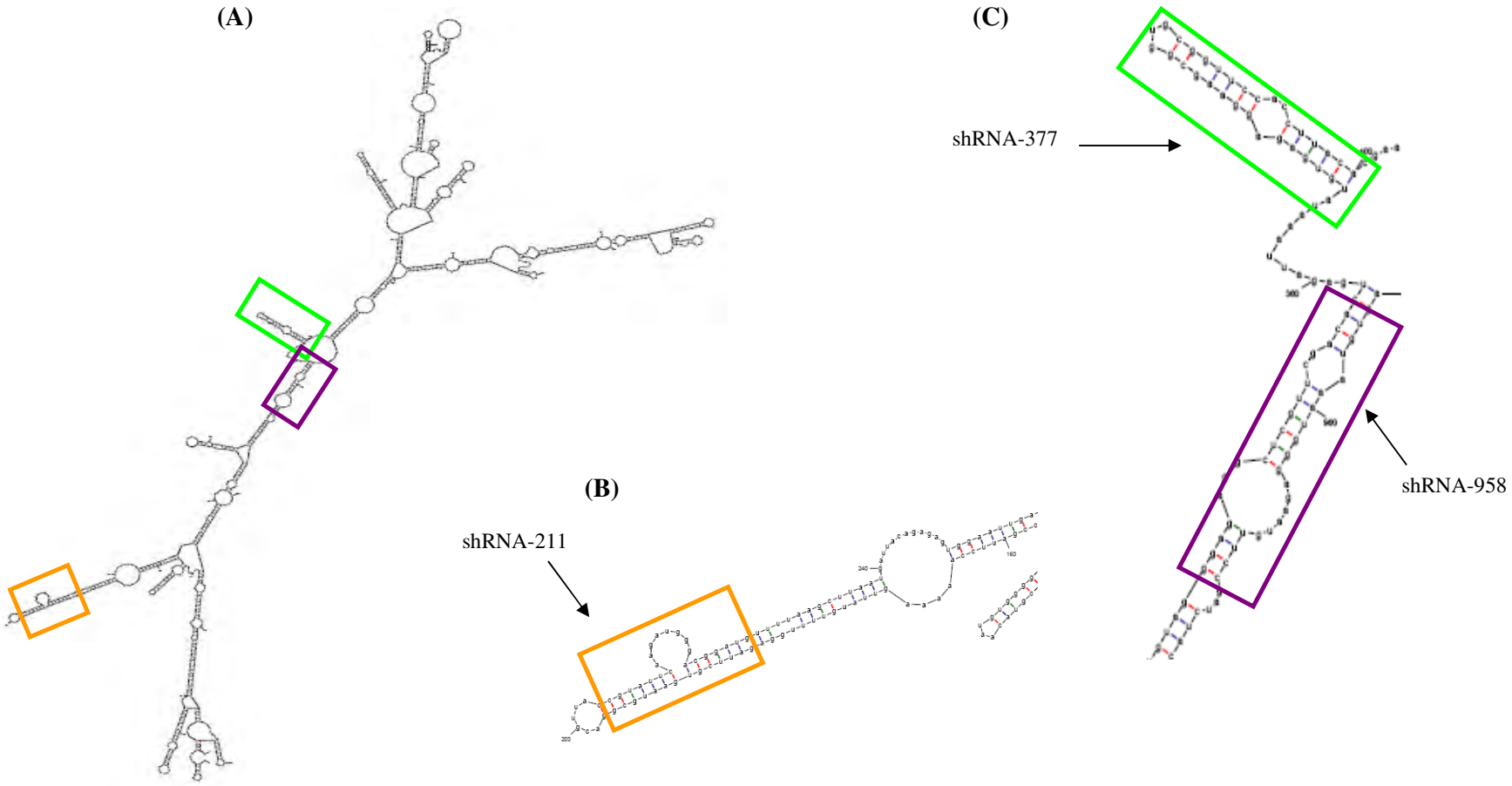

Fig. 3.2 Local secondary structure of the AHSV-9 NS2 mRNA (A) and close-up views (B and C) of the shRNA target sites, as predicted by the MFOLD software program (Zuker, 2003). The regions targeted by the NS2-directed shRNAs are boxed in orange (shRNA-211), green (shRNA-377) and purple (shRNA-958).

3.2.3.1 Construction of recombinant pSUPER plasmids

3.2.3.1.1 Preparation of annealed oligonucleotide inserts

The oligonucleotides were each dissolved in sterile nuclease-free water to a concentration of 3 mg/ml and 1 µl of the complementary forward and reverse oligonucleotides were mixed with 48 µl of annealing buffer (100 mM NaCl; 50 mM HEPES; pH 7.4). The mixture was incubated at 90°C for 4 min, then at 70°C for 10 min and subsequently allowed to cool to room temperature. To enable cloning of the annealed oligonucleotide inserts into the dephosphorylated pSUPER vector DNA, the 5' ends of the oligonucleotide duplexes were phosphorylated. The reaction mixture contained 500 ng of annealed oligonucleotides, 10 U of T4 polynucleotide kinase (10 U/µl; Fermentas), 10 mM ATP, 1 × reaction buffer and UHQ water to a final volume of 20 µl. The reaction mixtures were incubated at 37°C for 30 min and then stored at -20°C until needed.

3.2.3.1.2 Preparation of pSUPER vector DNA

The pSUPER vector DNA (3 µg) was sequentially digested at 37°C by first digesting the vector DNA with 10 U of *Hind*III for 1 h, followed by digestion with 10 U of *Bgl*II for 2 h. The digested vector DNA was subsequently dephosphorylated by addition of 6 U of Shrimp alkaline phosphatase (1 U/µl; Fermentas), 4 µl of 1 × reaction buffer and UHQ water to a final volume of 40 µl. The reaction mixtures were incubated at 37°C for 30 min and then at 65°C for 15 min to inactivate the phosphatase enzyme.

3.2.3.1.3 Cloning of shRNA duplexes into pSUPER vector DNA

The dephosphorylated vector DNA and phosphorylated annealed oligonucleotides were ligated at 4°C for 16 h in a final volume of 10 µl, which contained 1 µl of a 10 × DNA ligase buffer and 1 U of T4 DNA ligase (1 U/µl; Roche Diagnostics). The ratio of the insert to vector DNA was in excess of 5:1. Competent *E. coli* DH5α cells were subsequently prepared and transformed with the ligation reaction mixture, after which the cells were plated onto LB agar supplemented with ampicillin. Since the pSUPER vector lacks a *lac*Z' marker gene, transformants were randomly selected and extracted plasmid DNAs were screened by restriction enzyme digestion. The presence and integrity of the cloned insert DNA was furthermore verified by automated nucleotide sequencing procedures with the ABI-PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction kit v.3.1 (Perkin-Elmer Applied Biosystems), and primers T3 (5'-AATTAACCCTCACTAAAGGG-3') and T7 (5'-GTAATACGACTCACTATAGGG-3') as the sequencing primers. The derived recombinant plasmids were designated pSUPER-211, pSUPER-377, pSUPER-958 and pSUPER-Uneg.

3.2.3.2 Construction of recombinant pSUPER.Retro.Puro plasmids

A recombinant pSUPER.Retro.Puro plasmid was constructed with the aim of integrating the most efficient AHSV-9 NS2-directed shRNA into the genome of BHK-21 cells. To construct recombinant plasmid pSUPER.RT-377, the shRNA-377 expression cassette was recovered from plasmid pSUPER-377 by digestion with both *Eco*RI and *Xho*I and the agarose gel-purified 316-bp insert DNA was subsequently cloned into identically prepared pSUPER.Retro.Puro vector DNA. A similar approach was followed to construct recombinant plasmid pSUPER.RT-Uneg. The derived recombinant plasmid DNAs were characterized by restriction enzyme digestion.

3.2.4 Plasmid DNA extraction and purification

3.2.4.1 Plasmid DNA extraction

Large-scale plasmid extractions were performed as described by Sambrook *et al.* (1989), with the following modifications. Five ml of an overnight culture was inoculated into 500 ml of LB broth supplemented with the appropriate antibiotic, and incubated overnight at 37ºC. The bacterial cells were harvested by centrifugation at 5 000 rpm for 15 min in a Sorvall® RCB Plus centrifuge and washed once with 76 ml of 1 × STE buffer (10 mM Tris-HCl; 1 mM EDTA; 0.1 M NaCl; pH 8). The cell pellet was suspended in 40 ml of Solution I (50 mM glucose; 10 mM EDTA; 25 mM Tris; pH 8), and incubated on ice for 30 min. The cells were lysed by 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. Following incubation on ice for 60 min, the insoluble aggregate that formed was removed by centrifugation at 10 000 rpm for 15 min. The plasmid DNA-containing supernatant was recovered and filtered through Whatman® filter paper, after which the plasmid DNA was precipitated by addition of two volumes of 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 of UHQ water and 25 ml of ice-cold 7.5 M NH_4OAc was added. Following incubation on ice for 30 min, the high-molecular-weight RNA precipitate was removed by centrifugation at 10 000 rpm for 15 min. The plasmid DNA was subsequently precipitated from the supernatant by ethanol precipitation, as described above. The DNA pellet was washed with 70% ethanol, dried under vacuum and suspended in 300 µl of 1 × TE buffer (10 mM Tris-HCl; 1 mM EDTA; pH 8).

3.2.4.2 Purification of plasmid DNA

Plasmid DNA was extracted and purified using a commercial kit (Plasmid Midi Kit, Qiagen) according to the manufacturer's instructions. Alternatively, plasmid DNA obtained by large-scale extractions was purified by centrifugation in CsCl-ethidium bromide density gradients, as described by Sambrook *et al.* (1989). The volume of the large-scale recombinant plasmid DNA solution was

adjusted to 1 ml with 1 × TE buffer. An amount of 3.234 g of CsCl was added to 2 ml of 1 × TE buffer and the solution was incubated at 30°C to facilitate 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 solution of ethidium bromide was then added. The gradients were centrifuged at 38 000 rpm for 40 h in a Beckman SW55Ti rotor, using a Sorvall® Ultra Pro80 centrifuge. The tubes were viewed under UV light and the lower plasmid DNA-containing band was removed by inserting a needle, connected to a syringe, into the tube just below the lower band. The plasmid DNA solution was transferred to a 50-ml Greiner tube and diluted with 4 volumes of 1 × TE buffer. The ethidium bromide was removed from the solution by repeated extraction with an equal volume of water-saturated *n*-butanol. The lower aqueous phase was recovered, the volume adjusted to 4 ml with 1 × TE buffer and the plasmid DNA, free of RNA and protein contaminants, was subsequently precipitated by addition of 2 volumes of 96% ethanol. Following incubation at 4°C for 1 h, the precipitated plasmid DNA was recovered by centrifugation at 8 000 rpm for 20 min, rinsed with 70% ethanol and vacuum-dried prior to being suspended in 300 µl of 1 × TE buffer.

3.2.4.3 Quantification of plasmid DNA concentration

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

3.2.5 Plasmid-mediated gene silencing assays in BHK-21 cells

3.2.5.1 Cells and virus

Baby hamster kidney (BHK)-21 cells were propagated and maintained as monolayers in 75-cm^2 tissue culture flasks, and cultured in Minimum Essential Medium (MEM) (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 in the presence of 5% CO_2. African horse sickness virus serotype 9 (AHSV-9), used in virus challenge assays, was provided by Mr. F. Weege (Department of Genetics, University of Pretoria). AHSV-9 was propagated in confluent BHK-21 monolayers using a low-passage stock virus as inoculum.

3.2.5.2 Co-transfection of BHK-21 cells

BHK-21 cells were co-transfected with reporter plasmid pCMV-NS2-eGFP and the respective recombinant pSUPER plasmids by means of lipofection, using Lipofectamine™ 2000 reagent (Invitrogen) according to the manufacturer's instructions. The day before transfection, cells were

trypsinized, diluted in fresh medium and seeded in the wells of a 24-well tissue culture dish to reach 60% confluency within 24 h of incubation at 37°C in the presence of 5% CO_2. For transfection, 800 ng of each plasmid DNA and 2 µl of the Lipofectamine™ 2000 reagent was separately diluted in 50 µl of MEM medium (lacking serum and antibiotics). Following incubation at room temperature for 5 min, the two solutions were mixed and incubated at room temperature for 20 min to allow the formation of DNA-lipofectamine complexes. Just prior to transfection of the BHK-21 cells, the pCMV-NS2-eGFP-lipofectamine complexes were mixed with that of pSUPER-211, pSUPER-377 or pSUPER-958. The BHK-21 cell monolayers were prepared for transfection by rinsing the cells three times with 500 µl of antibiotic-free MEM medium, but containing 2.5% (v/v) FBS. After addition of 300 µl of the same medium, the cells were overlayed with the DNA-lipofectamine complexes (200 µl). The tissue culture dishes were then incubated at 37°C for 24 h in a CO_2 incubator.

3.2.5.3 Analysis and quantification of NS2-eGFP expression in BHK-21 cells

BHK-21 cell monolayers were observed at 24 h post-transfection for NS2-eGFP protein expression on a Zeiss Axiovert 200 fluorescence 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 with a Nikon DMX1200 digital camera. For fluorometry, the BHK-21 cell monolayers were washed once with 1 × PBS (137 mM NaCl; 2.7 mM KCl; 4.3 mM Na_2HPO_4; 1.4 mM KH_2PO_4; pH 7.4) and then trypsinized by the addition of 20 µl of Trypsin Versene. Following incubation at 37°C for 15 min, the cells were collected by centrifugation at 5 000 rpm for 10 min, washed with 1 × PBS and suspended in 1 ml of 1 × PBS. The relative fluorescence was determined with a BioRad Versafluor™ fluorometer (excitation at 485-495; emission at 515-525 nm).

3.2.6 Cell transfection and virus challenge assays in BHK-21 cells

The day before transfection, 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. The BHK-21 cells were then transfected with the respective recombinant pSUPER plasmids using Lipofectamine™ 2000 reagent (Invitrogen). Briefly, 4 µg of the purified plasmid DNA and 10 µl of the Lipofectamine™ 2000 reagent were each diluted in 250 µl of MEM medium (lacking serum and antibiotics), incubated at room temperature for 5 min and then mixed. Following incubation at room temperature for 20 min, the DNA-lipofectamine complexes (500 µl) were overlayed on BHK-21 cells, which had been prepared for transfection as described above. The tissue culture dishes were then incubated at 37°C for 24 h in a CO_2 incubator. Subsequently, the transfection mixtures were removed and the cell monolayers rinsed three times with MEM medium (lacking serum and antibiotics), prior to being infected with AHSV-9 at a MOI of 1 p.f.u./cell. After 1 h of infection, the virus inoculum was removed and 2 ml of serum- and antibiotic-

free MEM medium was added to the cells. At 24 h post-infection, the cells were processed for RNA isolation, as described below.

3.2.7 Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

3.2.7.1 Primers

Primers used for relative quantification of specific mRNA transcripts by real-time PCR were designed based on the available nucleotide sequence for the AHSV-9 NS2 gene (GenBank Acc. no. M69090) and a cellular gene encoding β2-microglobulin (β2-MG; GenBank Acc. no. X17002). The primers were designed with DNAMAN v.4.13 (Lynnon Biosoft), and optimal primer pairs were analyzed and identified with PerlPrimer v.1.1.6 (Marshall, 2004). Each of the primers were subjected to a BLASTN analysis (available at http://www.ncbi.nlm.nih.gov/BLAST) to verify target sequence specificity. The primers, indicated in Table 3.2, were obtained from Operon Technologies.

Table 3.2 Primers used in semi-quantitative real-time PCR

Primer	Nucleotide sequence	Tm (°C)	Annealing position	Target mRNA
β2-MG-F	5' - AGTGGAGCTGTCAGATCTGTCCTTC - 3'	64.5	9-34	BHK β2-MG*
β2-MG-R	5' - TGACCACCTTGGGCTCCTTC - 3'	64.8	127-147	BHK β2-MG
NS2F-9	5' - GATGTGCGCGAGATCACCTTG - 3'	66.4	586-607	AHSV-9 NS2
NS2R-9	5' - TGTCTTCGTGCCACATCATCTG - 3'	64.5	668-689	AHSV-9 NS2

*β2-MG β2-microglobulin

3.2.7.2 Total RNA extraction

Total RNA was extracted from BHK-21 cells with the Aurum™ Total RNA extraction kit (BioRad) according to the manufacturer's instructions. Briefly, the culture medium was removed and the cells were rinsed once with 1 × PBS, trypsinized and then collected by centrifugation at 5 000 rpm for 10 min. The cell pellets were suspended in 350 µl of the supplied lysis solution and lysed by vigorous pipetting before being transferred to an Eppendorf tube. After addition of an equal volume of 70% ethanol, the cell lysate was centrifuged through an RNA-binding column at 15 000 rpm for 30 s. The column was rinsed with wash solution, treated for 15 min at room temperature with RNase-free DNase I to remove contaminating chromosomal DNA, 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 primer pairs indicated in Table 3.2. 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 an appropriate DNA molecular weight marker.

3.2.7.3 cDNA synthesis

RNA was reverse transcribed using the QuantiTect® Reverse Transcription kit (Qiagen) according to the manufacturer's instructions. Prior to reverse transcription, the RNA preparations (10 µl) were each incubated at 42°C for 2 min with 1 × Genomic DNA Wipeout buffer (supplied in the kit) to eliminate any traces of contaminating genomic DNA. For reverse transcription of the RNA, 4 µl of 5 × Quantiscript® RT buffer, 1 µl of Quantiscript® Reverse Transcriptase and 1 µl of a RT primer mix (containing a mixture of oligo-dT and random primers) were added to the RNA preparation in a final volume of 20 µl. The reaction mixtures were subsequently incubated 42°C for 30 min, after which the enzyme was inactivated by heating to 95°C for 3 min. The cDNA was stored at -20°C until required.

3.2.7.4 Semi-quantitative real-time PCR

Semi-quantitative real-time PCR was performed using the QuantiTect™ SYBR® Green PCR kit (Qiagen) and the LightCycler® 1.5 system (Roche Diagnostics). Each reaction mixture (20 µl) contained 1 µl of the reverse transcription reaction mixture, 10 pmol of each of the gene-specific sense and antisense primer and 10 µl of 2 × QuantiTect™ SYBR® Green PCR master mix (containing 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 45 cycles of denaturation at 94°C for 15 s, 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 DNA was omitted. 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 specific amplification, melt curve analysis of the amplified products was performed with the LightCycler® v.3.5.3 software package (Roche Diagnostics) according to the manufacturer's protocol, and the amplicons were also analyzed by agarose gel electrophoresis in the presence of an appropriate DNA molecular weight marker.

3.2.7.5 Data analysis

Data were analyzed with the Relative Expression Software Tool (REST©) (Pfaffl *et al.,* 2002), where the relative expression of target (NS2) normalized to an endogenous reference (β2-MG) in a NS2 shRNA-treated sample relative to an experimental control (control shRNA-treated, AHSV-infected cells), is given by $R = (E_{target})^{\Delta CPtarget\ (control\ -\ sample)}/(E_{ref})^{\Delta CPref\ (control\ -\ sample)}$. In this formula, R represents the relative expression ratio of the target gene, E is the PCR efficiency, ΔCP is the crossing point difference of an unknown sample versus a control sample, and ref represents a reference gene. The LightCycler® software was used to determine CP values.

3.2.8 Construction of stable shRNA-expressing BHK-21 cell lines

A kill curve analysis was initially performed to determine the minimum concentration of puromycin required to kill 95% of untransfected BHK-21 cells within five days of incubation. For this purpose, BHK-21 cell monolayers at 90% confluency in 25-cm^2 flasks were cultured with different concentrations of puromycin ranging between 1-10 µg/ml. An untreated BHK-21 cell monolayer was included in the analysis as a positive control for cell viability. The flasks were incubated at 37°C for five days in a CO_2 incubator and were examined daily by microscopy to monitor cell viability. The results of this analysis indicated that the minimum concentration of puromycin required to kill 95% of the BHK-21 cells on the fifth day was 7 µg/ml.

To select for stable transfectants, BHK-21 cells were subsequently seeded in 25-cm^2 tissue culture flasks to reach 60% confluency within 24 h of incubation at 37°C in a CO_2 incubator. The cells were then transfected with the recombinant pSUPER.RT-377 and pSUPER.RT-Uneg plasmids using Lipofectamine™ 2000 reagent, as described previously (Section 3.2.5), except that 8 µg of plasmid DNA and 20 µl of Lipofectamine™ 2000 reagent was each separately diluted in 500 µl of MEM medium (lacking serum and antibiotics). At 24 h post-transfection, the culture medium was aspirated and the cells washed three times with 2 ml of MEM medium (lacking serum and antibiotics). Following addition of 5 ml of complete MEM medium (containing 5% [v/v] FBS and antibiotics) and 7 µg/ml puromycin, the tissue culture flasks were incubated at 37°C for five days in a CO_2 incubator. Following incubation, the cell monolayers were rinsed once with complete MEM medium, and 5 ml of the same medium and 7 µg/ml puromycin was then added. The tissue culture flasks were incubated at 37°C in a CO_2 incubator until a confluent monolayer had formed (*ca.* five days). Untransfected BHK-21 cell monolayers grown in the presence of puromycin were included as a control to determine the efficiency of the transfection and selection, while monolayers grown in the absence of puromycin were used to monitor cell viability.

3.2.9 Virus challenge assay in shRNA-expressing BHK-21 cell lines

The day before virus infection, the BHK-Uneg and BHK-377 cells, obtained as described above, were trypsinized, diluted in fresh MEM medium and 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. After incubation, the cell monolayers were rinsed three times with serum- and antibiotic-free MEM medium and then infected with AHSV-9 at a MOI of 1 p.f.u./cell. After 1 h of infection, the virus inoculum was removed and 2 ml of serum- and antibiotic-free MEM medium was added to the cells. At 24 h post-infection, the cells were processed for semi-quantitative RT-PCR analysis as described in Section 3.2.7.

3.2.10 PCR analyses of shRNA-expressing BHK-21 cell lines

3.2.10.1 Primers

The BHK-377 and BHK-Uneg cell lines were analyzed for the presence of integrated copies of the shRNA-377 and shRNA-Uneg expression cassettes by PCR analyses. Primers In-F and In-R were designed to anneal to the 5' LTR and 3' ΔLTR of the viral vector DNA, respectively, which flank the expression cassettes. Additional primers were designed to amplify the ampicillin resistance gene present on the pSUPER.Retro.Puro vector backbone. These primers, Ramp-F and Ramp-R, were used to determine whether the vector DNA or expression cassette only had integrated into the genome of the respective BHK-21 cell lines. The primers, indicated in Table 3.3, were obtained from Inqaba Biotechnical Industries.

Table 3.3 Primers used in PCR analyses of genomic DNA extracted from BHK-377 and BHK-Uneg

Primer	Nucleotide sequence	Tm (ºC)	Annealing position
In-F	5' - GGCCAAGAACAGATGGTC - 3'	59.9	173
In-R	5' - CACAAGTCGGATGCAACTG - 3'	60.1	3160
Ramp-F	5' - ATCCGCTCACAATTCCAC - 3'	57.6	3423
Ramp-R	5' - GCCTATATCGCCGACATC - 3'	59.9	6149

3.2.10.2 PCR amplification

The PCR reaction mixtures (50 µl) contained 50 ng of chromosomal DNA, 1 × PCR buffer, 1.5 mM $MgCl_2$, 50 pmol of each of the sense and antisense primer, 2.5 mM of each dNTP and 1 U of SUPERTHERM *Taq* DNA polymerase (Southern Cross Biotechnology). PCR was performed in a Perkin-Elmer GeneAmp® 2400 thermal cycler. The cycling profile consisted of an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 52°C for 45 s and elongation at 72°C for 5 min. For all of the analyses, UHQ water served as a negative control, while chromosomal DNA extracted from the wild-type and recombinant BHK-21 cells provided sample template DNA. Following PCR amplification, 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.

3.3 RESULTS

3.3.1 Construction of recombinant pSUPER shRNA delivery plasmids

Towards developing a plasmid DNA vector-based RNAi assay whereby AHSV-9 NS2 expression could be silenced in BHK-21 cells, the pSUPER shRNA delivery vector was selected for use in this study. The vector directs intracellular synthesis of shRNA transcripts using the RNA polymerase III H1-RNA gene promoter and has been used successfully to mediate efficient and specific down-regulation of gene expression of different cellular and viral genes (Brummelkamp *et al.*, 2002a; Shlomai and Shaul, 2003; Karlas *et al.*, 2004; Huang *et al.*, 2006; Fan *et al.*, 2006). To construct the recombinant pSUPER vectors, complementary oligonucleotides corresponding to different NS2-specific shRNA sequences and a control non-silencing shRNA sequence (Table 3.1) were annealed and cloned into pSUPER vector DNA that had been digested with both *Bgl*II and *Hind*III, as described under Materials and Methods (Section 3.2.3.1). Following transformation of competent *E. coli* DH5α cells, the plasmid DNA extracted from randomly selected transformants was characterized by restriction digestion.

Since the *Bgl*II site of the vector DNA is destroyed upon successful ligation with the insert DNA, the plasmid DNA was digested with both *Eco*RI and *Hind*III, which flank the transcription unit (Fig. 3.3A). Digestion of the plasmid DNA with these restriction enzymes resulted in the excision of a DNA fragment of 295 bp, which corresponds in size with the H1 promoter region (227 bp) together with the cloned oligonucleotide insert (68 bp). In contrast, digestion of the parental pSUPER vector DNA with both *Eco*RI and *Hind*III excised only the 227-bp H1 promoter region (Fig. 3.3B).

To confirm that no extraneous mutations were introduced into the oligonucleotides during their chemical synthesis, the integrity of the cloned oligonucleotide insert DNA was verified by nucleotide sequencing. No nucleotide differences were observed between the cloned oligonucleotide insert DNA and their intended AHSV-9 NS2 target regions (results not shown). The recombinant plasmids harbouring oligonucleotide inserts corresponding to AHSV-9 NS2-specific shRNA sequences were designated pSUPER-211, pSUPER-377 and pSUPER-958, whereas recombinant plasmid pSUPER-Uneg harboured an oligonucleotide insert of which the corresponding shRNA does not display homology to known viral and cellular genes, and thus served as a non-silencing control.

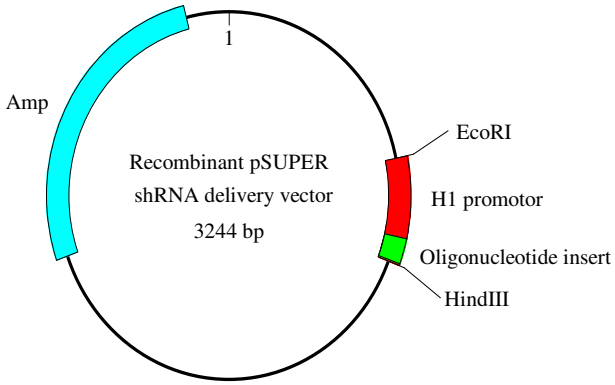

Fig. 3.3A Plasmid map of a recombinant pSUPER vector, harbouring oligonucleotides (indicated in green) corresponding to either AHSV-9 NS2 gene sequences or a control non-silencing sequence.

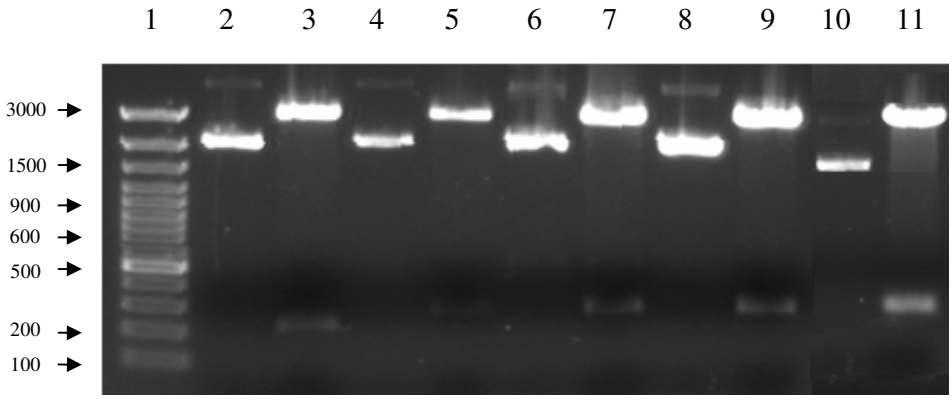

Fig. 3.3B Agarose gel electrophoretic analysis of recombinant pSUPER plasmids. Lane 1, DNA molecular weight marker; lane 2, uncut parental pSUPER vector DNA; lane 3, parental pSUPER vector DNA digested with *Eco*RI and *Hind*III; lane 4, uncut pSUPER-211 plasmid DNA; lane 5, pSUPER-211 plasmid DNA digested with *Eco*RI and *Hind*III; lane 6, uncut pSUPER-377 plasmid DNA; lane 7, pSUPER-377 plasmid DNA digested with *Eco*RI and *Hind*III; lane 8, uncut pSUPER-958 plasmid DNA; lane 9, pSUPER-958 plasmid DNA digested with *Eco*RI and *Hind*III; lane 10, uncut pSUPER-Uneg plasmid DNA; lane 11, pSUPER-Uneg plasmid DNA digested with *Eco*RI and *Hind*III. The sizes of the DNA molecular weight marker, Gene Ruler™ 100-bp DNA Ladder Plus (Fermentas), are indicated to the left of the figure in base pairs.

3.3.2 Silencing of NS2-eGFP protein expression by shRNAs in BHK-21 cells

To determine whether the designed candidate shRNAs could efficiently reduce expression of NS2 in BHK-21 cells, the constructed recombinant pSUPER plasmids were co-transfected into BHK-21 cells together with the previously constructed pCMV-NS2-eGFP reporter plasmid. Since the AHSV-9 NS2 coding region was fused in-frame with that of the eGFP gene, shRNA-mediated cleavage of the chimeric mRNA would lead to reduced expression of the reporter and thus provide a means whereby the efficiency of gene silencing can be analyzed at the protein level by fluorescence microscopy and quantitatively by fluorometry.

To investigate, BHK-21 cells were co-transfected with pCMV-NS2-eGFP in the absence or presence of pSUPER-211, pSUPER-377 or pSUPER-958. The BHK-21 cell monolayers were examined for NS2-eGFP protein expression by fluorescence microscopy at 24 h post-transfection. The results obtained (Fig. 3.4A) indicated extensive fluorescence of cells transfected with pCMV-NS2-eGFP. However, expression of the NS2-eGFP protein was markedly reduced in cells co-transfected with the recombinant pSUPER plasmids expressing the respective NS2-directed shRNAs. To determine and compare the efficiency of the different candidate shRNAs to silence NS2-eGFP gene expression, the cells were subsequently harvested and the expression of NS2-eGFP was analyzed quantitatively by fluorometry (Fig. 3.4B). The results indicated that in cells co-transfected with pSUPER-211, pSUPER-377 or pSUPER-958, expression of the NS2-eGFP protein was reduced by 90%, 86% and 80%, respectively. Together, these data suggested that the shRNAs directed to the AHSV-9 NS2 gene could induce a significant reduction in the level of NS2 protein.

3.3.3 Silencing of AHSV-9 NS2 mRNA expression by shRNAs in BHK-21 cells

Since RNAi functions by silencing gene expression at a post-transcriptional level, the effect of the different candidate shRNAs on NS2 mRNA expression in AHSV-9 infected BHK-21 cells was subsequently examined. To investigate, BHK-21 cell monolayers were transfected with the recombinant pSUPER plasmids expressing NS2-directed shRNAs or a control non-silencing shRNA. After incubation for 24 h, the transfected cells were infected with AHSV-9. At 24 h post-infection, total RNA was isolated from the cells and subjected to reverse transcription followed by semi-quantitative real-time PCR. In this study, β2-microglobulin (β2-MG) was used as endogenous reference gene for data normalization and for calculation of fold changes in NS2 transcripts with the REST© software programme (Pfaffl *et al.*, 2002).

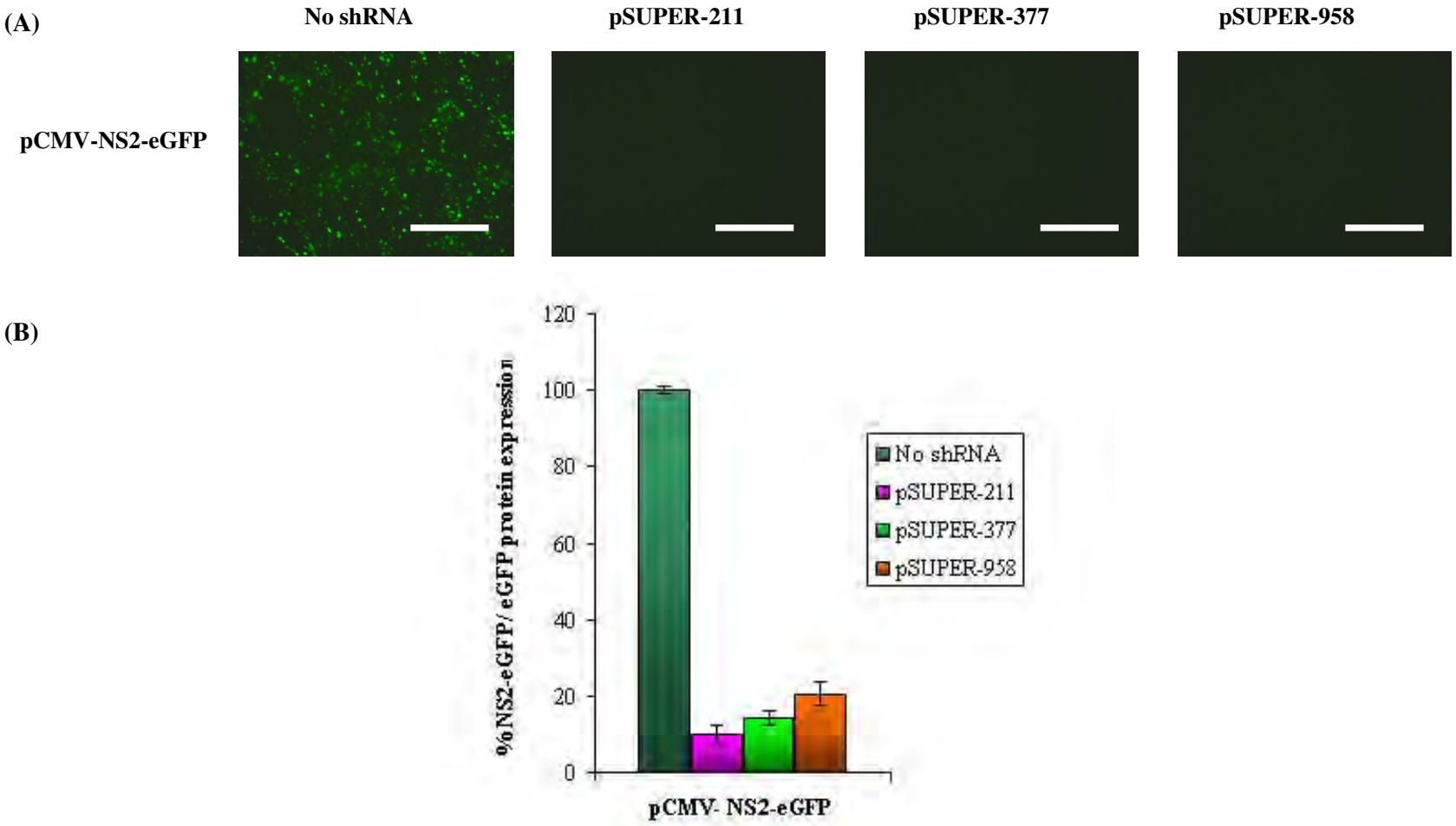

Fig. 3.4 Silencing of NS2-eGFP protein expression in BHK-21 cells. (A) Fluorescent micrographs of cells transfected with reporter plasmid pCMV-NS2-eGFP and co-transfected with the NS2 shRNA-expressing recombinant plasmids pSUPER-211, pSUPER-377 and pSUPER-958. At 24 h post-transfection, representative fields were photographed. Magnification, 10×. Bar = 500 µm . (B) Cells were analyzed for NS2-eGFP expression by fluorometry and the level of fluorescence relative to the cells transfected with the pCMV-NS2-eGFP reporter plasmid was quantified. Data are the means ± standard deviation (S.D.) from three independent experiments.

The data, presented in Fig. 3.5, indicated that not all of the NS2-directed shRNAs reduced NS2 mRNA expression in the AHSV-9 infected BHK-21 cells. In BHK-21 cells transfected with pSUPER-211, no reduction in NS2 mRNA transcripts could be observed. Indeed, the level of NS2 mRNA transcripts was similar to that in cells transfected with the control pSUPER-Uneg shRNA delivery plasmid. In contrast to BHK-21 cells transfected with pSUPER-211, NS2 mRNA expression was, however, reduced in BHK-21 cells transfected with pSUPER-377 and pSUPER-958. The results indicated that shRNA-377 and shRNA-958 induced a 3.4- and 2.1-fold reduction in NS2 mRNA transcripts, respectively, as compared with cells transfected with the control plasmid pSUPER-Uneg.

Despite the results obtained for pSUPER-211 being contradictory to that obtained in the preceding section, it should be noted that the amplification specificity of the semi-quantitative real-time PCR was verified by agarose gel electrophoresis and a single amplicon of the expected length for each target was obtained (103 bp for NS2; 138 bp for β2-MG) (results not shown). Furthermore, to confirm its reproducibility, melt curve analysis was performed and the efficiency of the semi-quantitative real-time PCR was also calculated. Serial dilutions, in triplicate, of the cDNA prepared from mock-transfected cells, AHSV-9 infected cells, as well as cells transfected with the respective recombinant pSUPER plasmids and then infected with AHSV-9 were subjected to semi-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_{NS2} = 1.75 and $E_{\beta 2\text{-MG}}$ = 1.95) were close to the theoretic maximum and optimum efficiency of *E* = 2, thus indicating that the semi-quantitative real-time PCR had a high level of reproducibility and sensitivity.

3.3.4 Time-course analysis of the inhibitory effect of shRNA-377 on NS2 mRNA expression in AHSV-9 infected BHK-21 cells

It has been reported previously that the first viral proteins in BTV-infected BHK-21 cells can be detected between 2-4 h post-infection, after which viral protein synthesis increases until 11-13 h and then it slows down until cell death (Huismans, 1979). Thus, to evaluate the time-course of the inhibitory effect of shRNA-377 on the expression of AHSV-9 NS2 mRNA, BHK-21 cells were individually transfected with pSUPER-377 and pSUPER-Uneg. At 24 h post-transfection, the cells were infected with AHSV-9 and then harvested at 6, 12 and 24 h post-infection. The cells were subsequently processed for determination of fold changes in NS2 mRNA levels by semi-quantitative real-time RT-PCR analyses.

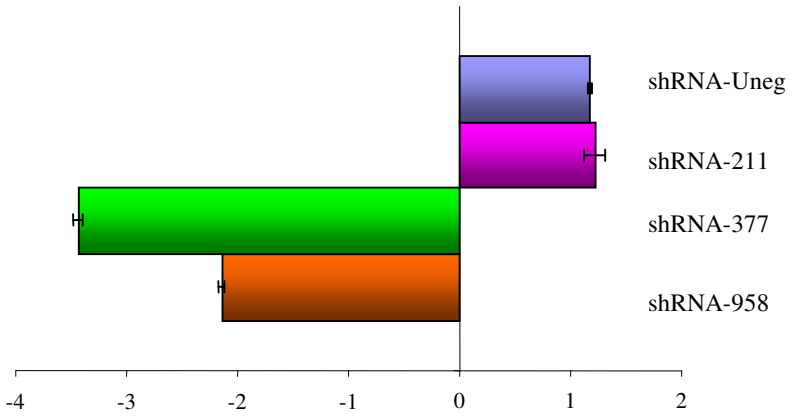

Fig. 3.5 Semi-quantitative real-time RT-PCR analysis for relative quantification of NS2 mRNA expression in AHSV-9 infected BHK-21 cells. Fold changes in NS2 mRNA expression levels were calculated by relative quantification of NS2 mRNA in cells transfected with recombinant pSUPER plasmids expressing NS2-directed shRNAs (shRNA-211, shRNA-377 and shRNA-958) as compared to cells transfected with pSUPER-Uneg expressing a control non-silencing shRNA (shRNA-Uneg). Real-time RT-PCR for β2-microglobulin gene transcripts was included in the assays as endogenous reference and used for data normalization of NS2 mRNA fold changes. Data are shown as the means ± S.D. of three samples.

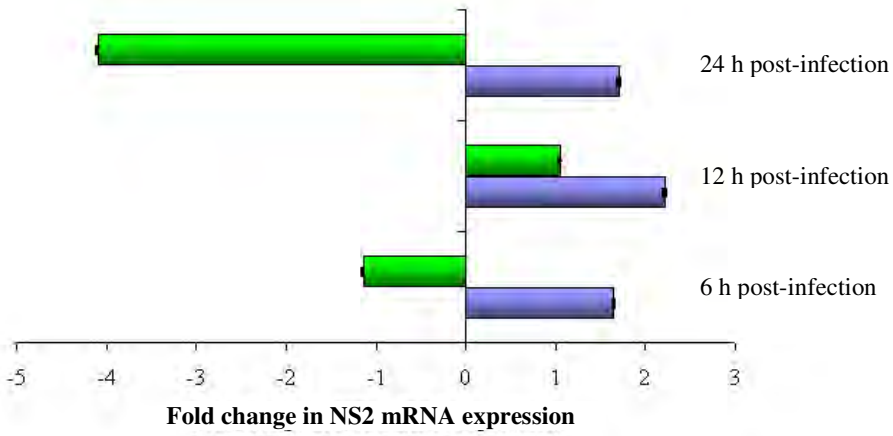

Fig. 3.6 Time-course analysis of the inhibitory effect of shRNA-377 on the expression of NS2 mRNA in AHSV-9 infected BHK-21 cells. The fold changes in NS2 mRNA expression levels at 6, 12 and 24 h post-infection were determined by semi-quantitative real-time RT-PCR analysis, as described in the legend to Fig. 3.5. Data are shown as the means ± S.D. of three samples.

Compared to cells transfected with the control pSUPER-Uneg shRNA delivery plasmid, the results indicated that shRNA-377 reduced NS2 mRNA expression 1.2-fold in the transfected cells at 6 h post-infection. However, at 12 h post-infection, no significant reduction in NS2 mRNA transcripts could be observed, although NS2 mRNA expression was *ca.* half of that observed for cells transfected with the control plasmid pSUPER-Uneg. Notably, at 24 h post-infection, shRNA-377 induced a 4.1-fold reduction in NS2 mRNA transcripts (Fig. 3.6). These results therefore indicated that inhibitory effects on NS2 gene expression could be observed during the early and late stages of viral infection, but these effects were greatly diminished when viral protein expression was at its maximum (12 h).

3.3.5 Silencing of AHSV-9 NS2 gene expression in shRNA-expressing BHK-21 cell lines

Since retroviral vectors can provide an opportunity to stably integrate an RNAi cassette into the recipient cell genome (Root *et al.*, 2006; Musiyenko *et al.*, 2007), the possibility of using the shRNA-377 expression cassette for construction of a continuous BHK-21 cell line, in which the AHSV-9 NS2 gene is stably suppressed, was subsequently explored. For this purpose, the pSUPER.Retro.Puro vector was used that had been derived from a self-inactivating murine stem cell virus plasmid (pMSCV) (Brummelkamp *et al.*, 2002b).

3.3.5.1 Construction of recombinant pSUPER.Retro.Puro plasmids

Although recombinant pSUPER plasmids, harbouring oligonucleotide inserts corresponding to the NS2-directed shRNA-377 and control non-silencing shRNA-Uneg, had been constructed previously, it was not possible to simply excise and reclone the shRNA oligonucleotide inserts into the pSUPER.Retro.Puro vector. This was as a consequence of the cloning strategy used, since successful cloning of the oligonucleotide inserts destroyed the *Bgl*II site of the recombinant plasmids. Therefore, the shRNA-377 and shRNA-Uneg oligonucleotide inserts, together with the upstream H1 promoter region, were recovered from plasmids pSUPER-377 and pSUPER-Uneg, respectively, as 316-bp *Xho*I - *Eco*RI DNA fragments and cloned into identically digested pSUPER.Retro.Puro vector DNA. Following transformation of competent *E. coli* DH5α cells, plasmid DNA was extracted from randomly selected transformants and characterized by restriction enzyme digestion. Recombinant plasmids from which a 316-bp insert DNA was excised (Fig. 3.7), thus confirming that the H1 promoter region of pSUPER.Retro.Puro was replaced successfully with the shRNA transcriptional units obtained from the recombinant pSUPER plasmids, were selected for further use, and designated pSUPER.RT-377 and pSUPER.RT-Uneg, respectively.

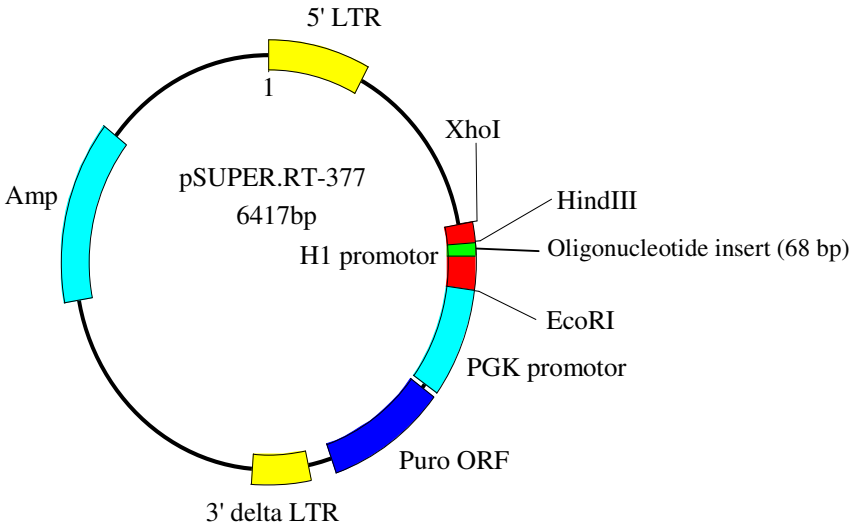

Fig. 3.7A Plasmid map of the recombinant plasmid pSUPER.RT-377.

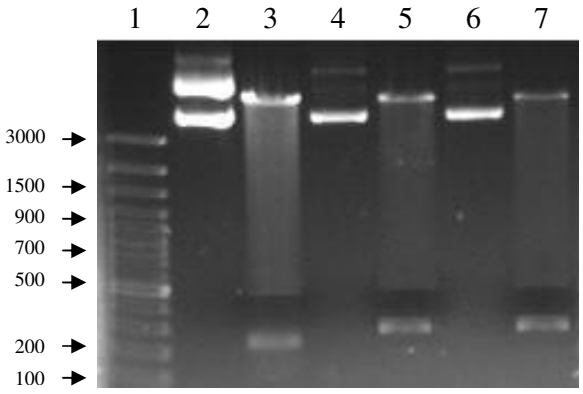

Fig. 3.7B Agarose gel electrophoretic analysis of recombinant pSUPER.Retro.Puro plasmids. Lane 1, DNA molecular weight marker; lane 2, uncut parental pSUPER.Retro.Puro vector DNA; lane 3, parental pSUPER.Retro.Puro vector DNA digested with *Xho*I and *Eco*RI; lane 4, uncut pSUPER.RT-Uneg plasmid DNA; lane 5, pSUPER.RT-Uneg plasmid DNA digested with *Xho*I and *Eco*RI; lane 6, uncut pSUPER.RT-377 plasmid DNA; lane 7, pSUPER.RT-377 plasmid DNA digested with *Xho*I and *Eco*RI. The sizes of the DNA molecular weight marker, Gene Ruler™ 100-bp DNA Ladder Plus (Fermentas), are indicated to the left of the figure in base pairs.

3.3.5.2 Silencing of AHSV-9 NS2 mRNA expression in BHK-377 and BHK-Uneg cells

BHK-21 cell monolayers were transfected with pSUPER.RT-377 or pSUPER.RT-Uneg and transfectants were selected by 7 µg/ml puromycin, a concentration that produced the optimal killing curve on untransfected BHK-21 cells in previous tests (Materials and Methods, Section 3.2.9). After selection, the respective cell lines, designated BHK-377 and BHK-Uneg, were seeded in tissue culture dishes and subsequently infected with AHSV-9 at a MOI of 1 p.f.u./cell. At 24 h post-infection, the abundance of NS2 mRNA in the infected cells was examined by semi-quantitative real-time RT-PCR, as described in the preceding sections. The data, presented in Fig. 3.8, indicated that compared to BHK-Uneg cells, NS2 mRNA expression was reduced in BHK-377 cells and a 1.3-fold reduction in NS2 mRNA was obtained. These results suggested that the BHK-377 cell line was capable of silencing AHSV-9 NS2 gene expression through degradation of the NS2 mRNA transcripts, albeit at a much reduced efficiency compared with the results obtained using the recombinant pSUPER-377 shRNA delivery vector (Figs. 3.5 and 3.6).

3.3.6 PCR analyses of the BHK-377 and BHK-Uneg cell lines

To determine whether copies of the shRNA-377 and shRNA-Uneg expression cassettes were indeed integrated into the genome of the respective BHK cell lines, different PCR assays were performed. Since integration of these cassettes into the cell genome occurs randomly and based on a lack of sequence information regarding the genome sequences that may flank the insertion sites, primer pairs were designed based on the available nucleotide sequence of the pSUPER.Retro.Puro vector (Fig. 3.9A). Primers In-F and In-R were designed to anneal to sequences within the 5' LTR and 3' ΔLTR, respectively, and would amplify a 3.055-kb product that corresponds in size with the integration cassette. In addition, primers Ramp-F and Ramp-R were designed to anneal to the pSUPER.Retro.Puro vector backbone and would result in amplification of a 2.727-kb product that harbours the vector-borne ampicillin resistance cassette. Thus, if the integration cassettes were indeed present in the genomes of the respective shRNA-expressing BHK cell lines, this primer pair would not be expected to yield an amplicon. For the PCR analyses, genomic DNA extracted from BHK-377 and BHK-Uneg cells that had been maintained and propagated in the presence or absence of puromycin selection were used as template DNA. The recombinant pSUPER.Retro.Puro plasmids were included as positive controls, while genomic DNA from BHK-21 cells was included as a negative control. The results of these analyses are shown in Fig. 3.9B.

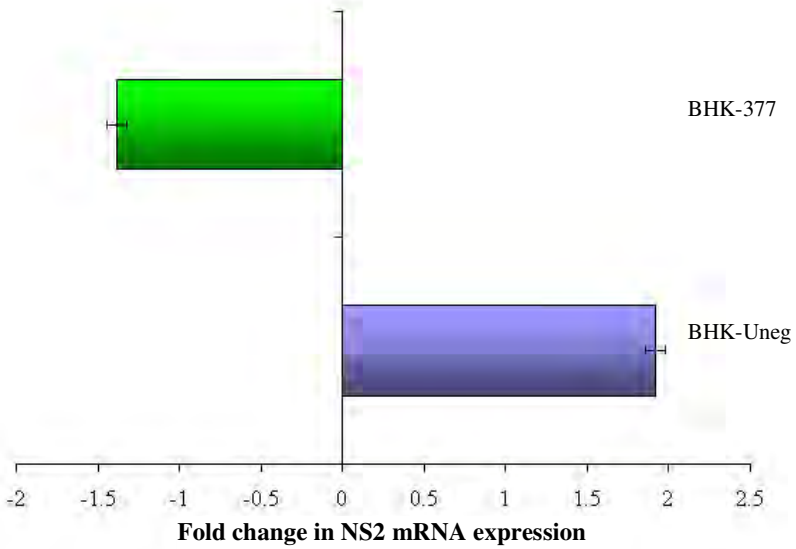

Fig. 3.8 Semi-quantitative real-time RT-PCR analysis for relative quantification of NS2 mRNA expression in AHSV-9 infected BHK-377 and BHK-Uneg cells. The fold change in NS2 mRNA expression levels was calculated by relative quantification of NS2 mRNA in BHK-377 cells expressing the NS2-directed shRNA-377 as compared to BHK-Uneg cells expressing the control non-silencing shRNA (shRNA-Uneg). Real-time RT-PCR for β2-microglobulin gene transcripts was included in the assays as endogenous reference and used for data normalization of NS2 mRNA fold changes. Data are shown as the means ± S.D. of three samples.

By making use of genomic DNA extracted from BHK-377 or BHK-Uneg, which had been propagated in the presence of puromycin selection, as templates for PCR, a single band of *ca.* 3 kb was obtained with primers In-F and In-R (Fig. 3.9B, lanes 6 and 7, respectively), while a band of *ca.* 2.7 kb was obtained with primers Ramp-F and Ramp-R (lanes 15 and 16, respectively). The sizes of the bands corresponded with the sizes of the amplicons obtained when recombinant plasmids pSUPER.RT-377 and pSUPER.RT-Uneg were used as templates in the PCR reactions. As expected, no products were amplified when genomic DNA of BHK-21 cells was used as template in the respective PCR reactions (Fig. 3.9B, lanes 5 and 14, respectively). Notably, no amplification products were observed when genomic DNA extracted from BHK-377 and BHK-Uneg, which had been propagated in the absence of puromycin selection, were used as templates in PCR reactions together with primers In-F and In-R (Fig. 3.9B, lane 8 and 9, respectively) or with primers Ramp-F and Ramp-R (lanes 17 and 18, respectively).

Taken together, these results therefore indicated that integration of the respective shRNA-expression cassettes into the genome of BHK-21 cells did not occur and consequently, that the recombinant shRNA-expressing plasmids were maintained in an extrachromosomal state when the monolayers were grown in the presence of puromycin. However, in the absence of puromycin antibiotic selection pressure the extrachromosomal plasmids were lost from the cells, probably as a consequence of repeated cell division during growth of the cells to form confluent monolayers.

3.4 DISSCUSION

RNA interference (RNAi) is an evolutionary conserved gene silencing mechanism that has emerged as a powerful genetic approach whereby gene function in mammalian cells can be analyzed (Hammond *et al.*, 2001b; McManus and Sharpe, 2002; Shen, 2004; Dykxhoorn and Lieberman, 2005). It has been demonstrated that RNAi-mediated gene silencing can be obtained in cultured mammalian cells by transfection of chemically synthesized siRNA molecules (Caplen *et al.*, 2001; Elbashir *et al.*, 2001b) or by endogenous expression of short hairpin RNA (shRNA) that are subsequently processed into functional siRNA by cellular enzymes (Brummelkamp *et al.*, 2002a; Paddison *et al.*, 2002). Several DNA-based plasmid vectors and viral vectors have been developed that constitutively transcribe shRNA from the

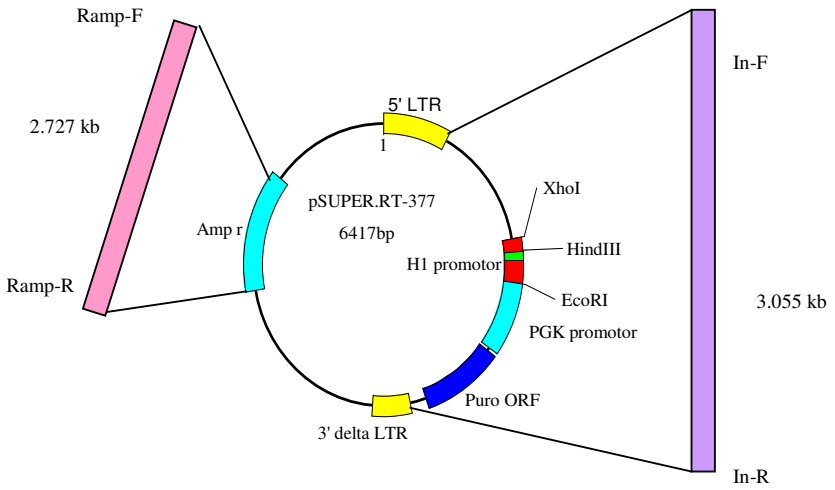

Fig. 3.9A Plasmid map of the recombinant plasmid pSUPER.RT-377, indicating the annealing positions of primers In-F and In-R, as well as primers Ramp-F and Ramp-R.

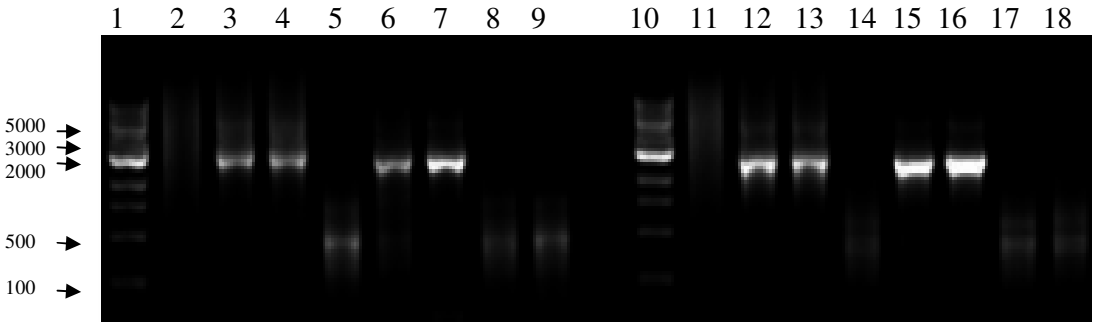

Fig. 3.9B Agarose gel electrophoretic analysis of the amplification products following PCR analyses of BHK-377 and BHK-Uneg. As controls, PCR reactions lacking template DNA (lanes 2 and 11), PCR reactions containing recombinant plasmids pSUPER.RT-Uneg (lanes 3 and 12) and pSUPER.RT-377 (lanes 4 and 13) as template DNA, as well as PCR reactions containing genomic DNA from BHK-21 cells as template DNA (lanes 5 and 14) were included. Genomic DNA extracted from BHK-377 (lanes 6 and 15) and BHK-Uneg cells (lanes 7 and 16), which had been propagated with puromycin selection, as well as genomic DNA from BHK-377 (lanes 8 and 17) and BHK-Uneg (lanes 9 and 18) cells, which had been propagated without the puromycin antibiotic selection, were included in the analyses. Lanes 2 through 9 indicate amplicons obtained with primers In-F and In-R, whereas lanes 11 through 18 indicate amplicons obtained with primers Ramp-F and Ramp-R. The sizes of the DNA molecular weight marker (lanes 1 and 10), GeneRuler™ Express DNA Ladder 100-5000 bp (Fermentas), are indicated to the left of the figure in base pairs.

RNA polymerase III U6 or H1 promoters and have been reported to result in stable and efficient suppression of target genes (Brummelkamp *et al.*, 2002b; Paddison *et al.*, 2002; Sui *et al.*, 2002; Xia *et al.*, 2002; Rubison *et al.*, 2003; Grimm *et al.*, 2005; Wu *et al.*, 2005). Consequently, in this investigation, a DNA vector-based approach was used to silence expression of the AHSV-9 NS2 gene in BHK-21 cells.

One caveat of siRNA design is that not all RNA duplexes will cleave their target with efficiency and much effort has gone toward identifying a set of rules for selecting an effecitve siRNA target site within a gene (Elbashir *et al.*, 2001a; Caplen *et al.*, 2001; Khvorova *et al.*, 2003; Reynolds *et al.*, 2004; Ui-Tei *et al.*, 2004). Despite these developments, there are presently no reliable methods to select effective siRNA sequences without empirical testing. In this study, three 19-nt target sequences from different regions in the coding region of the AHSV-9 NS2 gene were thus selected using different siRNA design algorithms. The designed sense and complimentary antisense oligonucleotides for each target site were subsequently synthesized, annealed and cloned into the pSUPER shRNA delivery plasmid such that the shRNA was transcribed from the constitutive H1 RNA promoter of the vector.

To screen and identify a functional shRNA that can efficiently inhibit AHSV-9 NS2 gene expression, the previously constructed pCMV-NS2-eGFP reporter plasmid was co-transfected with the respective pSUPER shRNA delivery plasmids into BHK-21 cells. The results indicated that NS2-eGFP expression was markedly reduced in these cells compared to cells transfected with the reporter plasmid only, and fluorometry analysis indicated that the level of inhibition mediated by the shRNAs were in excess of 80% (Fig. 3.4). Since the hallmark of RNAi is the degradation of targeted mRNA transcripts, the potential of the NS2-directed shRNAs to reduce the level of NS2 transcripts in AHSV-9 infected BHK-21 cells was subsequently investigated. The results indicated that of the three shRNAs used, two (shRNA-377 and shRNA-958) interfered with NS2 mRNA expression to differing extents, while the third (shRNA-211) had no apparent effect on NS2 mRNA production (Fig. 3.5). Although such variation has been reported extensively (Ge *et al.*, 2003; Kapadia *et al.*, 2003; Vickers *et al.*, 2003; Wang *et al.*, 2004; Lambeth *et al.*, 2007), the reasons for the differences in gene silencing are not well understood. Nevertheless, a number of hypotheses have been proposed in the literature, including binding of viral and/or cellular proteins on the mRNA that may cause positional effects (Holen *et al.*, 2002), the local secondary and tertiary structure of the targeted mRNA that may effect the accessibility of the RNAi effector molecules (Bohula *et*

al., 2003; Kretschner-Kazemi Far and Sczakiel, 2003; Vickers *et al.*, 2003; Luo and Chang, 2004; Westerhout and Berkhout; 2007) and the thermodynamic properties of the RNAi effector molecules that play a role in its stability and strand bias in duplex unwinding and retention by RISC (Khvorova *et al.*, 2003; Schwartz *et al.*, 2003).

Interestingly, although shRNA-211 efficiently suppressed expression of the NS2-eGFP reporter, it did not induce a reduction in the AHSV-9 NS2 mRNA population. This silencing activity of shRNA-211 appears to mimic that of microRNAs (miRNAs), which due to mismatches of a small number of base pairs (2-3 bp) leads to translational inhibition instead of mRNA degradation (Doench *et al.*, 2003; Saxena *et al.*, 2003; Scacheri *et al.*, 2004; Pasquinelli *et al.*, 2005). Nucleotide sequence analysis of the cloned oligonucleotide inserts in the recombinant pSUPER shRNA delivery plasmids, however, did not indicate any mismatches between the shRNA and the intended NS2 gene target sequences. Therefore, a more likely explanation for the result is that shRNA-211 might have been ineffective because of changes to the secondary structures identified in the target region (Fig. 3.2) compared to that formed by the NS2 gene when fused to the eGFP gene (Fig. 3.1). Although the use of reporter systems such as that used in this investigation is generally accepted as a method of screening candidate sh/siRNAs (Yokota *et al.*, 2003; Werk *et al.*, 2005; Stassen *et al.*, 2007), the data obtained in this invetigation questions the reliability of such systems. A similar concern has also been noted by de los Santos *et al.* (2005) and Lambeth *et al.* (2007), although further and more comprehensive analysis is required.

The most efficient NS2-directed shRNA (shRNA-377) was subsequently selected and its inhibitory effect on AHSV-9 NS2 mRNA expression was analyzed in a time-dependant manner. The results indicated that AHSV-9 NS2 mRNA expression was suppressed most efficiently at 24 h post-infection in BHK-21 cells transfected with pSUPER-377 and to a much lesser extent at 6 h post-infection, but was inefficiently suppressed at 12 h post-infection (Fig. 3.6). Huismans (1979) reported that the NS2 protein in BTV-infected BHK-21 cells could be observed at 2 to 4 h post-infection after which an increase in the rate of its synthesis was noted until 11 to 13 h post-infection, followed by a decrease in NS2 synthesis until 26 to 28 h post-infection. This implies that NS2 is transcribed frequently up to 13 h post-infection and less frequently during the later stages of virus infection (Huismans and Verwoerd, 1973; Huismans; 1979). Since the kinetics of the RNAi machinery is not yet known and considering that the shRNA first needs to be transcribed and then processed by

Dicer into siRNA-like molecules prior to incorporation into RISC for degradation of homologous mRNA transcripts, it may thus be that there is an insufficient amount of the RNAi effector molecules to effect efficient degradation of the high amount of NS2 mRNA transcripts present in the cells. However, as the rate of NS2 transcription decreases a more potent silecing effect can subsequently be observed at 24 h post-infection. Similar time-dependant gene silencing effects by vector-expressed shRNAs has been reported previously (Chen *et al.*, 2004; Choi *et al.*, 2005; Peng *et al.*, 2005).

The results obtained during the course of this investigation indicated that pSUPER vector-expressed shRNA-377 could suppress AHSV-9 NS2 mRNA expression in virus-infected BHK-21 cells. Despite careful optimization of the RNAi assay conditions (data not shown), differences in transfection efficiencies may nevertheless lead to uneven distribution of the vector DNA in cells and thus to variability in the shRNA-mediated gene silencing (*e.g.*, compare Figs. 3.5 and 3.6). To overcome these limitations and to achieve a persistant inhibitory effect, some groups have reported the use of plasmid or retroviral vectors to select for stable shRNA-expressing clones (Brummelkamp *et al.*, 2002b; Cao *et al.*, 2004; Liu *et al.*, 2004b; Root *et al.*, 2006; Musiyenko *et al.*, 2007). To investigate, the shRNA-377 transcriptional unit was recloned from plasmid pSUPER-377 into the self-inactivating pSUPER.Retro.Puro retroviral vector, which had been derived from murine stem cell virus (Brummelkamp *et al.*, 2002b). Following transfection of BHK-21 cells with the recombinant plasmid DNA, stable transfectants were selected by growth in the presence of puromycin. Subsequent analysis of AHSV-9 NS2 mRNA expression in the derived BHK-377 cell line indicated that the amount of NS2 mRNA was lower compared to that of the virus-infected BHK-Uneg cell line, which expressed a control non-silencing shRNA (shRNA-Uneg). Furthermore, it was established by PCR analysis that the respective shRNA-expression cassettes were not integrated into the cell genome but that the plasmid DNA was rather maintained extrachromosomally.

Notably, in contrast to this investigation in which a BHK-21 cell line was used, other studies reporting the use of pSUPER.Retro.Puro in gene silencing assays have been confined to human cell lines (Brummelkamp *et al.*, 2002b; Musiyenko *et al.*, 2007; Zhang and Samuel, 2007). Only Musiyenko *et al.* (2007) reported on the characterization of the stable transfectants and demonstrated that insertion of the plasmid occurred via homologous recombination between the 5' LTR of the plasmid and endogeous retroviral LTRs or

sequences of close similarity in various chromosomes of the HeLa cell genome. In addition, recombination was also noted between the plasmid-borne H1 promoter and chromosome sequences, as well as between plasmid sequences of no known function and fortuitous homologous sequences in various chromosomes (Musiyenko *et al.*, 2007). Thus, a plausible explanation for the lack of plasmid integration observed in this study may be that no homologous sequences are found in the chromosomes of BHK-21 cells. Moreover, the low level of AHSV-9 NS2 mRNA suppression observed in the BHK-377 cells (Fig. 3.8) may be a consequence of the pSUPER.Retro.Puro-377 plasmid being maintained in an extrachromosomal state. It can be expected that during each cell division the amount of extrachromosomal plasmid DNA and expressed shRNA-377 is diluted per cell and therefore the suppresive effect.

In conclusion, the results obtained in this part of the study provides evidence that plasmid DNA vector-expressed shRNA may be used to silence AHSV-9 NS2 gene expression in BHK-21 cells. Not only may it facilitate further studies of NS2 function, but new experimental approaches can be designed to study the function of other AHSV genes in the context of AHSV infection. Moreover, the results obtained also suggest that the technology can be adapted to analyzing gene function over an extended period of time through stable inhibition, albeit that further optimization of the methodology is required for this purpose.

CHAPTER FOUR

CONCLUDING REMARKS

RNA interference (RNAi) is a cellular pathway that induces degradation of target mRNA in a sequence-specific manner, leading to post-transcriptional silencing of gene expression (Fire *et al.*, 1998; Elbashir *et al.*, 2001a). In virology, RNAi has found application both as a research tool whereby virus gene function can be determined and loss-of-function phenotypes can be created, and as an antiviral therapeutic approach (McManus and Sharpe, 2002; Dykxhoorn *et al.*, 2003; Haasnoot *et al.*, 2003; Dorsett and Tuschl, 2004; Stram and Kuzntzova, 2006). In cultured mammalian cells, RNAi can be induced by directly applying synthetic siRNAs, pools of siRNA or plasmid DNA- and viral vector-expressed small RNA transcripts, including shRNAs and siRNAs (Brummelkamp *et al.*, 2002a, 2002b; Donze and Picard, 2002; Elbashir *et al.*, 2002; Scherer *et al.*, 2004; Wadhwa *et al.*, 2004; Xuan *et al.*, 2006). Of these approaches, DNA plasmids and viral vectors can purportedly be used to stably express shRNA for extended periods of time under the control of a RNA polymerase III promoter, such as U6 or H1 (Brummelkamp *et al.*, 2002a, 2002b; Miyagishi and Taira, 2002; Paddison *et al.*, 2002; Sui *et al.*, 2002; Shen *et al.*, 2003). Our research group has recently demonstrated the use of chemically synthesized siRNA as a means to silence expression of the AHSV-9 VP7 gene (Stassen *et al.*, 2007). To extend these studies and in keeping with the long-term goal of analyzing AHSV gene function in infected host cells, the aim of this investigation was essentially to develop a DNA vector-based RNAi assay whereby expression of the NS2 gene of AHSV-9 could be silenced in BHK-21 cells.

To determine whether shRNA can mediate silencing of AHSV-9 NS2 gene expression in BHK-21 cells, oligonucleotides encoding the desired shRNA sequences were synthesized, annealed and cloned into the pSUPER shRNA delivery vector downstream of the H1 promoter. Analyses of the respective vector-expressed shRNAs for their ability to suppress NS2-eGFP reporter gene expression and subsequently NS2 mRNA synthesis in AHSV-9 infected BHK-21 cells indicated that they differed in their gene silencing efficiency. Of the different NS2-directed shRNAs tested, shRNA-377 and shRNA-958 were effective in silencing both reporter gene and NS2 mRNA expression, whilst shRNA-211 was ineffective in silencing mRNA expression. These results highlighted different aspects that may require further attention in future.

A major problem in the RNAi field is the design of effective shRNAs. Since shRNAs are processed by Dicer intracellularly to siRNAs, it has been suggested that shRNA design be based on the design rules for siRNA (Amarzguioui *et al.*, 2005). However, studies have

shown that although a siRNA sequence can effectively suppress gene expression, a corresponding DNA-expressed shRNA may not perform equally (Peng *et al.*, 2005; Lambeth *et al.*, 2007). It therefore appears that the design criteria for siRNAs do not necessarily equate to effective shRNA design. In addition, Taxman *et al.* (2006) demonstrated that none of the algorithms frequently used for siRNA design showed a statistically significant ability to discriminate between effective and ineffective shRNA. In the absence of satisfactory shRNA design tools, the most efficient shRNA should therefore still be selected empirically and may thus require, depending on budgetary constraints, the screening of an increased number of candidate shRNAs.

Although chimeric target gene-eGFP reporter constructs have been used frequently to screen for effective si/shRNA (Yokota *et al.*, 2003; Werk *et al.*, 2005; Stassen *et al.*, 2007), it appears that exceptions do exist. In this investigation, the AHSV-9 NS2-directed shRNA-211 silenced NS2-eGFP protein expression, but not NS2 mRNA expression in AHSV-9 infected BHK-21 cells. Similar results have been reported by other groups (de los Santos *et al.*, 2005; Lambeth *et al.*, 2007). Consequently, alternative validation systems for the functional screening and identification of effective shRNAs may be considered in future. These involve construction of reporter plasmids in which the reporter gene is fused either with a truncated version of the full-length target gene (Wang *et al.*, 2004; Liu *et al.*, 2005; Qian *et al.*, 2005) or with a short synthetic DNA fragment containing an RNAi targeting sequence (Smart *et al.*, 2005; Hung *et al.*, 2006). The efficiency of the candidate RNAi effector molecules can be determined by fluorescent microscopy or fluorometry where eGFP was used as reporter, while enzyme assays can be used in instances where luciferase was used as reporter. Alternatively, flow cytometric analysis methods can also be considered. For this purpose, two reporter plasmids are used in which the shRNA directed against the target gene is cloned into a shRNA delivery vector that can also express eGFP, while the target gene is cloned in-frame with a DsRed reporter gene. Analysis of cells transfected with both the vectors not only indicate the ability of a cognate shRNA to silence its target gene, as evidenced by a decrease in the presence of red fluorescent cells, but expression of eGFP provides accurate data on the transfection efficiency (Ho *et al.*, 2006; Tong *et al.*, 2008).

Results obtained during the course of this investigation indicated that NS2 mRNA expression was silenced 6 h post-infection and most efficiently at 24 h post-infection in AHSV-9 infected BHK-21 cells. However, at 12 h post-infection, when viral protein synthesis is at its

maximum (Huismans, 1979), the RNAi effect was greatly diminished. This may have been due to the formation of transcriptionally active cores, containing one copy of each of the 10 dsRNA genome segments, which could initiate a second round of transcription, thereby resulting in an amplified second wave of viral protein synthesis. This interpretation is consistent with the active transcription of BTV viral genes occurring in cores derived from mature infectious virions (Van Dijk and Huismans, 1980; 1988) and with results reported for reovirus (Nibert and Schiff, 2001). Nevertheless, efficient silencing of NS2 gene expression at an early stage of virus infection and indeed throughout the infectious cycle would be important in order to prevent or ablate the formation of VIBs. Subsequent to undertaking this investigation, several reports demonstrated that efficient RNAi-mediated gene silencing can be obtained by *in vitro*-transcribed shRNA (Wang *et al.*, 2005; Vlassov *et al.*, 2007) or by synthetic shRNA (Kim *et al.*, 2005; Siolas *et al.*, 2005) that may be transfected directly into the cells. In these studies it was also reported that 27- to 29-mer shRNAs were more potent inducers of RNAi than 19-mer shRNAs (used in this investigation). The enhanced potency of the longer duplexes was attributed to enhanced Dicer-mediated processing of the precursors (Kim *et al.*, 2005). Therefore, rather than using plasmid DNA-expressed shRNA, the use of these alternative RNAi effector molecules, including siRNA, as a means to silence AHSV-9 NS2 gene expression may in future be investigated, albeit more costly.

Despite the advantages associated with the use of shRNA-expressing plasmid DNA vectors, *e.g.*, they are cheap to produce and provide a continuous expression of shRNAs, some shortcomings remain. In particular, as was also observed during the course of this investigation, transfection efficiency is often low and inconsistent (Peng *et al.*, 2005; Lambeth *et al.*, 2007). Although vectors with antibiotic resistance markers have been constructed that allow enrichment of cells harbouring the shRNA-delivery plasmid and therefore may compensate for low transfection efficiency, this process requires several weeks or months and is ultimately problematic due to potential clonal effects (Wadhwa *et al.*, 2004; Amarzguioui *et al.*, 2005). To overcome these problems, viral vectors, including retrovirus, adenovirus and lentivirus, have been employed (Brummelkamp *et al.*, 2002b; Rubinson *et al.*, 2003; Wadhwa *et al.*, 2004; Grimm *et al.*, 2005). In this investigation, an attempt was made to establish a continuous BHK-21 cell line that stably expresses the NS2-directed shRNA-377. The results indicated modest inhibition of AHSV-9 NS2 mRNA expression in the derived BHK-377 cell line and it was shown that the recombinant pSUPER.Retro.Puro retroviral vector was maintained extrachromosomally. These results are contradictory to those of Musiyenko *et al.*

(2007) whom reported stable integration of recombinant pSUPER.Retro.Puro plasmid DNA into the chromosomes of HeLa cells, using a similar approach to that described in this investigation. This discrepancy may, however, be due to the lack of homologous sequences in the genome of BHK-21 cells to allow for plasmid integration via the 5' LTR of the pSUPER.Retro.Puro vector. In contrast to transfection of cells with recombinant pSUPER.Retro.Puro constructs, Brummelkamp *et al.* (2002b) made use of a packaging cell line and used the harvested viral supernatants for cell infection. However, this approach could not be used in this investigation, as BHK-21 cells most likely lack a murine ecotropic receptor required for infection by the recombinant murine stem cell virus vector. To overcome these problems, the human HeLa cell line may in future experiments be used since it has been reported to be permissive for BTV and AHSV replication (Wirblich *et al.*, 2006) or, alternatively, a different viral vector could be used. The latter approach may be more applicable, since HeLa cells do not resemble the natural vertebrate and invertebrate hosts of BTV and AHSV. Although lentiviral vectors have been reported to allow for persistent silencing of targeted gene expression through stable integration of shRNA-expression cassettes into the genomes of different human cell lines (Rubinson *et al.*, 2003; Qin *et al.*, 2003), it is not known whether such vectors will also integrate into the BHK-21 cell genome. Therefore, the use of non-integrating viral vectors, based on mutagenic adenoviruses (Xia *et al.*, 2002; Shen *et al.*, 2003; Zhao *et al.*, 2003; Grimm *et al.*, 2005), should be considered in future studies. These viral vectors are not only capable of infecting a broad spectrum of cell types, but high virus titres can be obtained in packaging cell lines and may therefore facilitate uniform and stable delivery of shRNA to the cells (Grimm *et al.*, 2005). In this regard, it is worth noting that Chen *et al.* (2006) demonstrated that treatment of mammalian cells with a replication-defective human adenovirus (Ad5), expressing shRNA against the 1D and 3D genes of foot-and-mouth disease virus (FMDV), totally protected the cells from homologous FMDV infection for up to 36 h post-challenge.

In conclusion, the results obtained during the course of this investigation supported a proof-of-concept that shRNA can inhibit AHSV-9 NS2 gene expression. However, there may be room for improvement regarding the method of delivery of the shRNA and in searching for the ideal sequence or region in NS2 that may yield the optimum activity. Nevertheless, this approach holds promise for evaluating directly the role of NS2 in the context of replication of AHSV in infected mammalian cells.

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