

**The characterisation of aspects related  
to the single stranded RNA binding  
ability of African horsesickness virus  
nonstructural protein NS2**

**by**

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**dedicated to my parents, Gert and Amanda Grobler**

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## SUMMARY

### **The characterisation of aspects related to the single stranded RNA binding ability of African horsesickness virus nonstructural protein NS2**

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African horsesickness virus (AHSV) is a member of the *Orbivirus* genus and part of the family *Reoviridae*. It is a double stranded RNA virus with ten genome segments that encode seven structural and four nonstructural proteins. AHSV nonstructural protein NS2, encoded by segment eight, is a single stranded RNA (ssRNA) binding protein. The formation of viral inclusion bodies (VIBs) as well as the selection and condensation of the ten different virus genome segments during encapsidation, are proposed functions of NS2. This study investigates the binding ability of NS2 to ssRNA by looking at the protein structure, as well as its affinity for virus specific mRNAs. NS2 has an  $\alpha$ -helix rich C-terminal region and  $\alpha$ -helixes are known to be involved in ssRNA binding. BTV NS2 has an  $\alpha$ -helix content of 69% and AHSV NS2 an  $\alpha$ -helix content of 47%. AHSV NS2 has a lower binding affinity for ssRNA than bluetongue virus (BTV) NS2. There thus seems to be a correlation between the  $\alpha$ -helix content of different NS2 proteins and their ssRNA affinity. In order to determine if the difference between the ability of AHSV NS2 and BTV NS2 to bind nonspecifically to poly(U)Agarose can be ascribed to differences in the  $\alpha$ -helix rich C-terminal of NS2, a chimeric NS2 protein was constructed that contained the  $\alpha$ -helix rich C-terminal of BTV

NS2 and the N-terminal of AHSV NS2. The binding affinity of the chimeric NS2 to poly(U)Agarose was compared to that of AHSV NS2 and BTV NS2 and it was found that it correlated well with that of AHSV NS2. The  $\alpha$ -helixes therefore do not seem to play a major role in ssRNA binding. The binding affinity of NS2 rather seems to be determined by the N-terminal of the protein. In order to investigate the specific affinity of AHSV NS2 for AHSV mRNAs, it was necessary to obtain mRNA transcripts with terminal ends identical to AHSV mRNAs. The AHSV genes were cloned into a transcription vector with an internal ribozyme. The 3' ends of the transcripts were generated by autolytic cleavage mediated by the ribozyme immediately downstream of the viral insert. The vector's promoter was constructed in such a way that the 5' ends of the genes were inserted at the plus one position for transcription. Full-length mRNA transcripts of four AHSV genes were obtained, the genes encoding NS2, NS3, VP6 and VP7. mRNA transcripts of NS3 and VP7 genes, lacking three to five terminal nucleotides at either the 5' or 3' ends, were also obtained. A nonspecific mRNA control was derived from an AHSV gene cloned in the wrong transcriptional orientation. Preliminary binding assays showed that NS2 has at least a nonspecific affinity for all these mRNAs. The fluctuation in the results also suggests that mRNA concentration may play a role in protein/mRNA interactions. We suggest that NS2 may play a role in encapsidation by binding nonspecifically to all the mRNAs in the infected cell and thus presenting it at the VIBs for selection and encapsidation.

## OPSOMMING

### **Die karakterisering van aspekte rakende die enkeldraad RNA bindings vermoë van perdesiekte virus nie-strukturele proteïen NS2**

deur

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Perdesiekte virus (PSV) is lid van die *Orbivirus* genus in die *Reoviridae* familie. Dit is 'n dubbeldraad RNA virus met tien genoom segmente wat vir sewe strukturele en vier nie-strukturele proteïene kodeer. PSV nie-strukturele proteïen NS2, gekodeer deur segment agt, is 'n enkeldraad RNA bindende proteïen. Die vorming van virus insluitliggaampies (VIBs) asook die seleksie en kondensasie van die tien virus genoom segmente tydens virus verpakking is die funksies wat vir NS2 voorgestel is. Hierdie studie ondersoek die enkeldraad RNA bindings vermoë van NS2 deur die proteïen se struktuur en affiniteit vir virus spesifieke boodskapper RNA (mRNA) te ondersoek. NS2 het 'n  $\alpha$ -heliks ryke C-terminale gebied en  $\alpha$ -helikse is bekend daarvoor dat hulle betrokke is by enkeldraad RNA binding. Bloutong virus (BTV) NS2 het 'n  $\alpha$ -heliks inhoud van 69% en PSV NS2 'n  $\alpha$ -heliks inhoud van 47%. PSV NS2 het 'n swakker affiniteit vir enkeldraad RNA as BTV NS2. Dit lyk dus of daar 'n korrelasie tussen die  $\alpha$ -heliks inhoud van die verskillende NS2 proteïene en hul affiniteit vir enkeldraad RNA kan wees. Om te bepaal of die verskille tussen die vermoë van PSV NS2 en BTV NS2 om nie-spesifiek aan poli(U)Agarose te bind, toegeskryf kan word aan die verskille in die  $\alpha$ -heliks ryke C-terminale gebiede van NS2, is 'n chimeriese NS2

proteïen gekonstrueer wat bestaan uit die  $\alpha$ -heliks ryke C-terminaal van BTV NS2 en die N-terminaal van PSV NS2. Die bindings affiniteit van die chimeriese NS2 aan poli(U)Agarose is vergelyk met dié van PSV NS2 en BTV NS2 en dit is gevind dat dit goed met dié van PSV vergelyk. Dit lyk dus nie of  $\alpha$ -helikse 'n belangrike rol in enkeldraad RNA binding speel nie. Dit lyk asof NS2 se bindings affiniteit eerder deur die N-terminaal van die proteïen bepaal word. Om die spesifieke affiniteit van PSV NS2 vir PSV mRNA te bepaal, was dit nodig om mRNA transkripte te produseer waarvan die ente identies is aan dié van PSV mRNAs. Die PSV gene is gekloneer in 'n transkripsie vektor met 'n interne ribosiem. Die 3' ente van die transkripte word gegeneer deur outolitiese snyding bemiddel deur die ribosiem, direk stroomaf van die virus invoegsel. Die vektor se promotor is so gekonstrueer dat die gene se 5' ente by die plus een posisie van transkripsie ingevoeg is. Vollengte mRNA transkripte van vier PSV gene is verkry, die gene wat vir NS2, NS3, VP6 en VP7 kodeer. mRNA transkripte van NS3 en VP7 gene, wat drie tot vyf terminale nukleotide aan óf die 5' óf die 3' ent kortkom, is ook verkry. 'n Nie-spesifieke mRNA kontrole was afkomstig vanaf 'n PSV geen gekloneer in die verkeerde transkripsionele oriëntasie. Voorlopige bindings ondersoek wys dat NS2 ten minste nie-spesifieke affiniteit vir al hierdie mRNAs toon. Fluktuasie in die bindings dui daarop dat mRNA konsentrasie moontlik 'n rol in proteïen/mRNA interaksie speel. NS2 mag 'n rol speel in verpakking deur nie-spesifiek aan die mRNAs in geïnfecteerde selle te bind en dit op so 'n manier by die VIBs vir seleksie en verpakking aan te bied.

## ABBREVIATIONS

aa	amino acid
AcAHSV-9.8	recombinant baculovirus expressing NS2 of AHSV-9
AcBTV-10.8	recombinant baculovirus expressing NS2 of BTV-10
AcNPV	<i>Autographa californica</i> nuclear polyhedrosis virus
AHSV	African horsesickness virus
amp	ampicillin
Arg	arginine
ARM	arginine-rich motif
ARV	avian reovirus
ATP	adenosine-5'-triphosphate
ATPase	adenosine-5'-triphosphatase
bp	basepairs
BHK	baby hamster kidney
BMV	brome mosaic virus
BSA	bovine serum albumin
BSMV	barley stripe mosaic virus
BTV	bluetongue virus
C	carboxyl
°C	degrees Celsius
CarMV	camation mottle carmovirus
ccc	covalently closed circular
cDNA	complementary DNA
cm	centimetre
CLP	core-like particle
cpm	counts per minute
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
ddH <sub>2</sub> O	deionised distilled water
dGTP	2'-deoxyguanosine-5'-triphosphate
dNTP	deoxynucleotidyl-triphosphate



dTTP	2'-deoxythymidine-triphosphate
dUTP	2'-deoxyuridyl-triphosphate
DEPC	diethyl pyrocarbonate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
ds	double stranded
DTT	1,4-dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediaminetetra-acetic acid
EHDV	epizootic haemorrhagic disease virus
ER	endoplasmic reticulum
<i>et al.</i>	<i>et alia</i> (and others)
EtBr	ethidium bromide
FCS	fetal calf serum
Fig.	figure
g	gram
G	guanosine
h	hour
HBV	human hepatitis B virus
HIV	human immunodeficiency virus
IBDV	infectious bursal disease virus
i.e.	<i>id est</i> (that is to say)
IPTG	isopropyl- $\beta$ -D-thiogalactopyranoside
kb	kilobasepairs
kDa, K	kilodalton
KH	K-homology
L	large
M	medium / molar
mA	milliampere
MCS	multiple cloning site
mg	milligram
min	minutes



ml	millilitre
mm	millimetre
mM	millimolar
MOPS	N(Morpholino)propanesulfonic acid
mRNA	messenger ribonucleic acid
MRV	mammalian reovirus
MW	molecular weight
N	amino
nm	nanometre
NS	nonstructural
NSP	nonstructural protein
NTP	nucleotidyl-triphosphate
NTPase	nucleotidyl-triphosphatase
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pfu	plaque forming units
PSB	protein solvent buffer
PSV	perdesiekte virus
RI	replication intermediate
RNA	ribonucleic acid
Rnase	ribonuclease
rNTP	ribonucleotidyl-triphosphate
rpm	revolutions per minute
RT	room temperature
s	seconds
S	Svedberg units / small / segment
SDS	sodium dodecyl sulphate
Sf	Spodoptera fugiperda
SFV	semliki forest virus
ss	single-stranded
TaMV	tamarillo mosaic virus

TEMED	N,N,N',N'-tetramethylethylenediamine
tet	tetracycline hydrochloride
T <sub>m</sub>	melting temperature
TMV	tobacco mosaic virus
Tris	Tris-hydroxymethyl-aminomethane
TSWV	tomato spotted wilt virus
µg	microgram
µl	microlitre
µm	micrometer
U	units
UHQ	ultra high quality ddH <sub>2</sub> O
UV	ultraviolet
V	volts
VIB	viral inclusion body
VLP	virus-like particle
VP	virus protein
VSV	vesicular stomatitis virus
v/v	volume per volume
WT	wild-type
WTV	wound tumor virus
w/v	weight per volume
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

## LIST OF BUFFERS

20 × MOPS	0.4 M N(MOPS), 100 mM NaAc, 20 mM EDTA, pH 7
20 × SSPE	3.6 M NaCl, 2 mM Na <sub>2</sub> EDTA, 0.2 M NaH <sub>2</sub> PO <sub>4</sub> , 0.2 M Na <sub>2</sub> HPO <sub>4</sub> , pH 7
1 × TAE	0.04 M Tris-acetate, 1 mM EDTA, pH8
1 × TE	10 mM Tris, 1 mM EDTA, pH 7.4
1 × TGS	25 mM Tris, 0.25 M glycine, 0.1% SDS
0.01 M STE-TX	0.01 M NaCl, 0.01 M Tris, 1 mM EDTA, pH 7.4, 0.5% Triton X-100 <sup>R</sup>
0.15 M STE-TX	0.15 M NaCl, 0.01 M Tris, 1 mM EDTA, pH 7.4, 0.5% Triton X-100 <sup>R</sup>
0.4 M STE-TX	0.4 M NaCl, 0.01 M Tris, 1 mM EDTA, pH 7.4, 0.5% Triton X-100 <sup>R</sup>
0.5 M STE-TX	0.5 M NaCl, 0.01 M Tris, 1 mM EDTA, pH 7.4, 0.5% Triton X-100 <sup>R</sup>
1 × PBS	137 mM NaCl, 2.7 mM KCl, 4.3 mM Na <sub>2</sub> PO <sub>4</sub> , 1.4 mM KH <sub>2</sub> PO <sub>4</sub> , pH 7.3
2 × PSB	0.125 M Tris pH 6.8, 4% SDS, 20% glycerol, 10% β-mercapto-ethanol

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## CHAPTER 1

### LITERATURE REVIEW

#### 1.1. INTRODUCTION

Both African horsesickness virus (AHSV) and bluetongue virus (BTV) are members of the *Orbivirus* genus of the *Reoviridae* family (Bremer *et al.*, 1990). BTV is the prototype virus of the *Orbivirus* genus (Thomas *et al.*, 1990). The described orbiviruses are classified into 12 different serological groups and each group has a different number of serotypes (Gorman, 1983) as is shown in Table 1. Nine different AHSV serotypes (Bremer *et al.*, 1990; Grubman & Lewis, 1992) and twenty four different BTV serotypes (Roy, 1989) have been identified from different parts of the world. AHSV causes fatal disease in horses and is transmitted to susceptible animals by biting midges (*Culicoides* spp.) (Burrage *et al.*, 1993; Martinez-Torrecuadrada *et al.*, 1994).

Table 1: Groups of Orbiviruses (Gorman & Taylor, 1985; Gorman, 1992)

Species	Number of Serotypes
African horsesickness	9
Bluetongue	24
Epizootic haemorrhagic disease	10
Equine encephalosis	6
Eubanangee	2
Palyam	15
Umatilla	4
Changuinola	7
Corriparta	3
Kemerovo	20
Warrego	2
Wallal	2

Although the nonstructural protein NS2 of AHSV is the main subject of my thesis, I will focus on both AHSV and BTV in my literature review as there is more information available on BTV than on AHSV. I will also refer to other viruses and their proteins where they have similar functions to BTV and AHSV proteins as it is possible that a parallel might be drawn between the functions of the different virus proteins. The investigation into the known functions of similar proteins in other viruses might give us a clue to direct our research in the correct direction.

## 1.2. VIRAL MORPHOLOGY

Virus replication is dependant on virus morphology. It is believed that NS2, although not a structural protein of the virus, plays a role in viral replication. In order to examine virus replication, it is first of importance to review what the virus looks like.

Although no common antigen to all orbiviruses has been found, members of the *Orbivirus* genus do have similar morphology and properties (Gorman, 1983). Like reoviruses and rotaviruses these are non-enveloped viruses with concentric protein shells (Verwoerd, 1969; Roy, 1992). Three morphologically distinct virus particles namely virions, cores and subcore particles have been identified. The different particles are obtained and the morphological features are revealed by stepwise removal of some of the major structural proteins of the virus (Huismans & Van Dijk, 1990).

The innermost shell, or the subcore (390 S), has icosahedral symmetry and is composed of one major protein namely VP3. There are 120 molecules of VP3 per virus. These molecules are arranged with icosahedral symmetry within the subcore layer, forming two sets (A and B) of 60 subunits each. Both the VP3A and VP3B subunits have a similar overall structure (Grimes *et al.*, 1998). This encloses three minor proteins (VP1, VP4 and VP6) and the 10 double-stranded RNA segments namely segments 1 to 10. These segments are divided into three groups, namely large (L) segments, L 1-3; medium (M) segments, M 4-6; and small (S) segments, S 7-10 (Verwoerd *et al.*, 1970; Huismans *et al.*, 1987c; Roy, 1989; Roy, 1992). Each RNA segment, except segment 10 - the smallest, encodes a single viral protein (Roy, 1992). Subcores are unstable and apparently lack transcriptase activity *in vitro* (Huismans *et al.*, 1987c; Roy, 1989). These subcore particles are characterised by a skeleton-like appearance with a hexagonally shaped outline,

resembling the subvirus particles of rotavirus (Huismans & Van Dijk, 1990). Located also on this subcore is a second major protein namely VP7 (Roy, 1989; Roy, 1992). The subcore with VP7 and its internal components is now called the core (470 S). These cores exhibit a well-defined structure under the electron microscope and are very stable (Huismans *et al.*, 1987c; Roy, 1989). The main morphological feature of the core particles of BTV is the presence of 32 distinct morphological units, or capsomeres with a circular configuration. These capsomeres are arranged in icosahedral symmetry with the triangulation number T-3. They are tube-like, hollow structures with an axial hole. This characteristic ring-like configuration of the capsomeres is the feature from which the genus name *Orbivirus* is derived (“orbis” meaning ring or circle) (Verwoerd, 1969; Verwoerd *et al.*, 1972; Eaton *et al.*, 1990; Huismans & Van Dijk, 1990).

The inner surface of the VP3 layer is relatively bland with some shallow, sculpted grooves and a few charged residues. The core possesses small pores at the icosahedral three-fold axes and larger openings at the five-fold axes. A simple rearrangement of the side chain of Arg 154 of VP3B would enlarge the three-fold pores. Although these might act as portals in the subcore, the trimers of VP7 tightly plug these holes in the complete core. The pores at the five-fold axes are unencumbered by molecules of VP7, but they are too small to allow exit of mRNA and can not be significantly enlarged by side-chain movements alone. Activation of the cores for transcription is therefore likely to be accompanied by conformational changes at the five-fold axes to open the pores further. There are crystallographic mobility parameters which show that the core is particularly flexible around the five-fold axes with the VP7 trimers riding upon the VP3 layer and acquiring further flexibility in the exposed upper domains. These pores are lined with four arginines from each five-fold-related VP3A, of which three are strictly conserved across the orbiviruses. These positively charged groups may steer the RNA electrostatically and may be important in stripping counter ions from any RNA entering or exiting the particle (Grimes *et al.*, 1998). The cores also have an active RNA transcriptase and it is here that the ten genome segments are transcribed into 10 mRNAs by the RNA polymerase (Huismans & Van Dijk, 1990; Loudon & Roy, 1992). Although the core particles show a greatly reduced infectivity for mammalian cells, they retain a high infectivity for the insect vector, indicating that core proteins can also mediate cell attachment and penetration (Grimes *et al.*, 1998).

The complete virion is composed of the core plus an outer capsid. The outer capsid consists of

two other major proteins namely VP2 and VP5. This outer capsid surrounds the icosahedral core, has a fuzzy appearance, is nonstructured and is very loosely bound to the inner-shell proteins (Verwoerd *et al.*, 1972; Gorman, 1983; Roy, 1989; Eaton *et al.*, 1990; Prasad *et al.*, 1992). The fuzzy appearance of the particles clearly distinguishes AHSV and the other orbiviruses from reovirus and rotavirus which have a more structured and clearly defined outer capsid layer (Huismans & Van Dijk, 1990). In vivo almost all virions are again uncoated to core particles immediately after infection (Huismans *et al.*, 1987c; Huismans & Van Dijk, 1990) as the mRNA-synthesising enzyme activities are not expressed by intact virus particles. It is only after the outer capsid shell is partially or completely removed that the RNA transcriptase is expressed (Joklik, 1981). According to Eaton *et al.* (1990) core particles do not reassociate with newly synthesised outer coat proteins and are not released from the cell as progeny virus.

In addition to the seven structural proteins the virus has four nonstructural proteins namely NS1, NS2, NS3 and NS3A. NS3 and NS3A are both products of the segment 10 gene (Roy, 1992).

The purified virion is composed of about 80% protein and 20% dsRNA. The base composition of the RNA has a G + C value of 42.4%. Important common features of all the dsRNA segments are that the 5'- and 3'-noncoding regions are relatively short, varying between 8 and 34 base pairs. A sequence of seven nucleotides at the 3' ends (CACUUAC) and six nucleotides at the 5' ends (GUUAAA) of all dsRNA is also conserved. There is no evidence that any ssRNA is associated with the virion (Fukusho *et al.*, 1989; Huismans & Van Dijk, 1990). The virion-associated RNA transcriptase, located in the central viral core, synthesises single-stranded (positive sense) RNA transcripts that act both as mRNA and as precursors for the synthesis of the double-stranded genomic RNAs (McCrae & Joklik, 1978; Bellamy & Both, 1990). Synthesis of ssRNA begins almost immediately after infection, even when inhibitors of protein synthesis are present. This suggests that in the early stages of the infection the parental virus genome is used for transcription, and no protein synthesis is required. Newly synthesised ssRNA is not incorporated into the virion, but is associated with ribosomes in the infected cell. From 5 hours after infection the rate of ssRNA synthesis increased, probably due to the transcription of progeny virus templates (Verwoerd & Huismans, 1972; Spence *et al.*, 1984). Evidence was presented by Foster *et al.* (1978) that the genome, when extracted under acidic conditions (pH 4.0) with SDS and phenol, is occasionally seen as an unfragmented continuous structure. In most cases, however, the genome has a rosette

configuration with loops that emanate from a central area that resembles a doughnut. These rosette patterns each contains ten loops of varying length. The relative size of each of the ten loops is of about the same order as has been reported for the individual genome segments. The most surprising aspect of these results is, however, that the SDS-phenol extraction did not cause fragmentation of the dsRNA, which would seem to exclude a protein as possible linker of the genome segments (Huisman & Van Dijk, 1990). Data from the orthoreoviruses suggest that transcription of all ten BTV segments can occur simultaneously within a single virus particle (Gillies *et al.*, 1971). There is also evidence from the cypoviruses that suggests that each genome segment may be specifically associated with a single transcriptase complex situated at one of the vertices of the intact icosahedral virus particle (Yazaki & Miura, 1980).

Gouet *et al.* (1999) reported on the highly ordered dsRNA genome of bluetongue virus as revealed by crystallography. According to them there is a particular organisation of the various components within the core. Such ordering appears to be imposed by chemically featureless grooves that form tracks for the RNA on the inside of the VP3 layer. Specific RNA/protein interactions are only evident at two points in the icosahedral asymmetric unit. This scarcity of specific interactions appears likely to facilitate the movement of RNA within the core, for example during transcription, while the counter ions presumably lubricate the RNA/VP3 interface and the interactions between adjacent RNA layers. The transcriptional complexes, which are nonsymmetric, lie along the five-fold axes of the virus, at the heart of the RNA spirals, and below the pores in the VP3 layer, which if slightly expanded from the resting structure, would allow the exit of the RNA. The N-terminal 50 residues of VP3A, situated closest to the five-fold axes, swing down, compared to their positions in the VP3B molecule where they are well ordered, and engage the transcription complex, holding at least some of the enzyme components in the correct orientation. VP4, which has capping activity, lies immediately within the VP3 layer, forming strong interactions with residues 307-328 of VP3A. This explain how the cores can cap exogenous RNA. Internal to VP4, stabilized in part by interactions with the N-terminus of VP3A, lies the monomeric VP1 viral transcriptase. The limited flexibility but considerable fluidity of the dsRNA within a liquid crystal structure probably allows the double helixes to be pushed, pulled and spun about their helical axes as required for transcription, while a level of organization is maintained by the VP3 layer and the high RNA concentration (Gouet *et al.*, 1999).

Assortment of RNA molecules occurs at the stage of ssRNA. The earliest class of immature reovirus particles possesses a ssRNA to dsRNA polymerase (a replicase), the dsRNA never exists in free, unencapsidated form within infected cells (Joklik, 1981) The translation of the mRNAs is controlled at several levels. First, there is control mediated by the 5'-terminal cap. The mRNAs that are transcribed during the first four hours or so are capped and methylated: they are translated efficiently in extracts of uninfected cells, but not in extracts of cells that are infected, whereas uncapped mRNA molecules are translated poorly in extracts of uninfected cells, but efficiently in extracts of infected cells. Second, there is profound control over the frequency with which the ten species of virus mRNA are translated, some species being translated much more frequently than others. The relative efficiencies of translation seems to be the same both early during the multiplication cycle, when the mRNAs are capped, and late, when they are not capped. It is likely that the major, if not the sole, mechanism for regulating the translation frequencies of the ten mRNA species, which exist in the same environment at the same time, resides in their sequence content (Joklik, 1981). The first virus specific proteins are detected 2-4 hours post-infection, the rate of synthesis increases to 11 hours post-infection, where it remains constant for a further 15 hours. Synthesis declines after 32 hours post-infection. (Huisman, 1979; Spence *et al.*, 1984).

Now that we know more about the virus morphology, it is also important to be familiar with its entry and escape from the host cells. Entrance and escape are very important aspects in the virus life cycle. If the virus cannot infect its host or cannot spread from one cell to another it will become extinct.

### **1.3. VIRUS ENTRY AND ESCAPE**

There are several reports describing the presence of BTV in intracellular vacuoles shortly after infection. The virus first binds to a receptor on the plasma membrane at a site characterised by the presence of clathrin. The clathrin-coated membrane surface grows as a 'coated pit' which eventually invaginates and detaches from the cell surface, yielding a coated vesicle. This rapidly loses the clathrin coat. The vesicles may then fuse with, or fuse together, to form a larger electronlucent, endocytic vesicle: the endosome. Endosomes lack the hydrolytic enzyme found in lysosomes but resemble the latter in having an acidic pH (Bellamy & Both, 1990; Eaton *et al.*, 1990). Most viruses appear to use the endosome as the site of entry into the cell cytoplasm. The

low pH triggers a fusion activity in the glycoprotein spikes of enveloped viruses or a surface protein of nonenveloped viruses. This results in either fusion of the viral membrane with the limiting membrane of the vesicles or the direct penetration of the nonenveloped virus or its genome through the membrane. Studies on BTV uptake into cells have revealed few virus-like particles in lysosomes. This suggests that BTV may enter the cytoplasm by penetration of the endosomal membrane. The morphology of BTV particles within endosomes indicates that the acidic conditions therein (approximately pH 5.0) initiates the removal of the outer capsid layer from the virus. Within 1 hour of infection, BTV is converted to core particles. The release of virus from endosomes and lysosomes is dependent on the low pH in these organelles. Raising the intraendosomal and lysosomal pH results in the failure of endocytosed virus particles to enter the cytoplasm (Eaton *et al.*, 1990).

Following the release from endosomes, core particles of BTV in the cytoplasm become associated with a matrix. The matrix often appears to form at one side and in contact with the core particle but eventually spreads around it. These structures are viral inclusion body (VIB) precursors and they are presumably formed by condensation of viral RNA transcribed from the core particle and viral proteins translated on ribosomes outside the VIB. Particles with bishelled morphology are observed at the periphery of VIB starting at approximately 6 hours post infection, indicating that virus particles are synthesised within these structures. The presence of an outer layer in progeny particles at the VIB periphery suggest that they contain at least some outer coat proteins and therefore may not be able to synthesize mRNA as activation of the BTV transcriptase activity requires removal of both outer coat proteins VP2 and VP5. Progeny virus made early in infection may be released from the cell and reenter via endocytosis. The core particles liberated into the cytoplasm presumably initiate new VIB formation. An increase in the number of VIBs may raise the probability of synthesising, within the cell, core-like particles with active transcriptases. The process whereby progeny virus particles reinfect cells effectively increase the multiplicity at which cells are infected with BTV (Eaton *et al.*, 1990). VIBs, virus-specified tubules, and virus particles are associated with the cytoskeleton in BTV-infected cells. Virus particles are attached to filaments singly, in groups, or in linear arrays. Several observations point to intermediate filaments as being the site of virus binding (Eaton *et al.*, 1987).



Large numbers of BTV particles are released from the cells by budding through the cell membrane and some are seen as virus particles with lipoprotein envelopes. However, most virus particles attached to the grid substrate outside the cell, are not enveloped. BTV particles may 'escape from' or penetrate their surrounding lipid envelope, some evidence for this comes from the finding of large accumulations of membrane-like material outside infected cells. There are also data that suggest that in addition to budding individual virus particles and aggregates may penetrate the plasma membrane of infected cells and be released as nonenveloped viruses. It was found that cell death and subsequent release of infectious virus do not contribute significantly to the titer of released virus at least up to 24 to 30 hours postinfection (Eaton *et al.*, 1990). AHSV, similar to BTV, is also released from infected cells by budding as well as by a mechanism resembling extrusion (Stoltz *et al.*, 1996).

The next important step is to find out in more detail about the virus itself. As the virus consists of at least ten proteins it is important to know what each protein's function is. It is far too easy to forget that the protein that one is concentrating on is functioning as part of a virus and does not stand alone. The fact that a virus only consists of a few proteins, compared to other organisms, makes it easier to determine the specific functions of each protein. In this chapter the virus's proteins will be discussed under four headings: The minor core proteins; The major core proteins; The outer structural proteins; The nonstructural proteins. In each case the BTV and AHSV proteins will be reviewed first before some analogous proteins of a few other viruses will be discussed briefly. As a lot more is known about some proteins that shows a resemblance with BTV and AHSV proteins, such as rotavirus proteins, it is important to be aware of this information as it can be utilised to direct our future research.

## **1.4. THE THREE MINOR CORE PROTEINS**

### **1.4.1. VP1**

The largest viral protein VP1 is encoded by the largest dsRNA segment (L1). It is present in a very low molar ratio in the virion (Roy, 1989). Hybridisation data have indicated that this gene is highly conserved among all the serotypes of a specific virus (Fukusho *et al.*, 1989). This protein is the prime candidate for the virion RNA-dependent RNA polymerase and functions as the viral

transcriptase (Urakawa *et al.*, 1989; Turnbull *et al.*, 1996; Patton & Chen, 1999). The RNA-dependent RNA polymerase is the key enzyme for the animal RNA viruses to transcribe their RNA genomes. Since there is no enzymatic activity of similar property in animal cells, the animal RNA viruses have evolved to encode this unique enzyme within the genome RNA (Banerjee & Chattopadhyay, 1990). VP1 is also known to bind nucleotides, specifically to the 3' end of viral mRNA (Patton & Chen, 1999). Its sequence has a high degree of similarity not only with various DNA and RNA virus polymerases, but also with other eukaryotic polymerase subunits (Roy, 1992). The BTV-associated transcriptase is activated by removal of polypeptides VP2 and VP5 from the virus particle (Van Dijk & Huismans, 1980).

Reconstruction experiments performed with purified recombinant protein have shown that although VP1 of rotavirus is the candidate RNA polymerase, the protein requires the presence of VP2, BTV VP3 analog, for replicase activity. VP1 specifically recognizes the 3' end of the gene 8 mRNA. The fact that VP1 has both nonspecific and specific RNA-binding activity was previously noted for this protein and is an expected feature of RNA polymerases, since they not only have to recognize a promoter but also must move along a DNA or an RNA template during RNA synthesis. The inability of VP1 to bind to dsRNA suggests that, for the RNA polymerase to initiate transcription the 5' end of the dsRNA templates must first undergo denaturation by a helicase (Patton & Chen, 1999).

#### **1.4.2. VP4**

VP4, encoded from the M4 segment, is probably the guanylyl transferase in the virus and is responsible for the capping and methylating of the 5' ends of the virus mRNAs during transcription (Fukusho *et al.*, 1989; Huismans & Van Dijk, 1990; Roy, 1992). Direct evidence for GTP binding by VP4 has been obtained by using a recombinant baculovirus that expresses VP4 and it has also been shown that a protein-GTP complex catalyses the capping of the mRNA (Roy, 1992; Turnbull *et al.*, 1996).

The orbivirus analog of VP4 in rotavirus, VP3, is the viral guanylyltransferase and therefore is responsible for capping of viral mRNAs (Kattoura *et al.*, 1992). VP3 is a component of early replication intermediates, and it and VP1 seem to be the first two structural proteins to associate

with viral mRNA during packaging and RNA replication. VP3 can bind to the N terminus of VP2, a region of the core shell protein to which VP1 and ssRNA are also known to bind and that is required for RNA replication. Rotavirus guanylyltransferase has affinity for ssRNA but not for dsRNA. The ssRNA-binding activity of VP3 is nonspecific and the protein has a preferential affinity for uncapped as opposed to capped ssRNA. This implies that, while VP3 may not recognize the 5'-terminal sequence of the RNA, it may recognize features of the 5' end that are associated with the presence or the absence of a cap structure, e.g., a 5'-terminal  $\gamma$ - or  $\beta$ -phosphate group (Patton & Chen, 1999).

### 1.4.3. VP6

The third minor protein, VP6, is encoded by segment 9 (S9). It is abundant in arginine, lysine and histidine residues and binds nonspecifically to nucleic acids (Loudon & Roy, 1992; Roy, 1992). The protein exhibits two domains (N- and C-terminal) separated by a glycine-rich region. Since VP6 is a minor component of the virion it can be expected that, like VP1 and VP4, it is a component of the mRNA polymerase complex. Baculovirus-expressed and BTV-derived VP6 have been shown to have a strong binding affinity to both ss- and dsRNA species even in the presence of SDS, indicating that its binding capability is independent of tertiary structures (Turnbull *et al.*, 1996). VP6 is capable of binding not only the double-stranded BTV RNA species and single-stranded BTV RNA transcripts, but also a nonviral dsDNA probe and a nonviral ssRNA probe (Roy *et al.*, 1990a). Sequence analyses of VP6 have revealed a motif common to helicases. Helicases are involved in the unwinding of double-stranded DNA or RNA prior to transcription and replication, a process energetically coupled to the hydrolysis of nucleoside 5'-triphosphates (NTPs). VP6 may also have a role in encapsidation of the RNA. The BTV VP6 amino acid sequence is highly conserved within a serogroup and through all serotypes (Roy *et al.*, 1990; Roy, 1992; Turnbull *et al.*, 1996; Bisailon & Lemay, 1997; Maree *et al.*, 1998b).

The reovirus  $\lambda 1$  protein is able to unwind double-stranded nucleic acids molecules in the presence of NTPs or dNTPs and is a NTPase/helicase.  $\lambda 1$  has affinity for dsRNA and for dsDNA and its nucleic acid-binding activity is independent of the nucleic acid sequence. It was shown that the first ten amino acids of the protein are sufficient for binding (Bisailon & Lemay, 1997).

Semliki forest virus (SFV) NSP2 has ATPase and GTPase activities, which are stimulated by ssRNAs. This protein has RNA-binding ability as well as a ssRNA-stimulated NTPase activity typical of RNA helicases (Rikkonen *et al.*, 1994). The C-terminal domain of hepatitis C virus NS3 have also been shown to possess RNA-stimulated nucleoside triphosphatase and RNA helicase activities (Gallinari *et al.*, 1998). Another helicase is poliovirus protein 2C. It has nucleoside triphosphatase activity but is resistant to guanidine. Poliovirus RNA replication is inhibited by millimolar concentrations of guanidine hydrochloride (Pfister & Wimmer, 1999).

The CI protein of tamarillo mosaic potyvirus (TaMV), is also a RNA helicase. It was found that the TaMV CI protein requires single-stranded regions of RNA to initiate binding. Stimulation of ATPase activity by single-stranded RNA presumably coincides with the RNA-associated enzyme translocating along the RNA molecule, in a manner similar to the DNA-stimulated translocation of *E. coli* DNA helicase along DNA. In this way, translocation of the TaMV CI protein along the RNA molecule would be facilitated by the RNA-stimulated hydrolysis of NTP. All NTPs are used for RNA helicase activity, without any significant preference (Eagles *et al.*, 1994). An important aspect of NTPase activity during virus replication may be to provide free energy for as variety of processes, including those of viral RNA binding by the replicase complex, the unwinding of nascent duplex RNA formed during virus replication, and the removal of the secondary structure presented on the template RNA (Cui *et al.*, 1998). The putative RNA helicases are involved in diverse cellular functions such as translation initiation, RNA splicing and transcription regulation (Eagles *et al.*, 1994).

## **1.5. THE MAJOR CORE PROTEINS**

### **1.5.1. VP7**

The VP7 protein is located on the surface of the core particles and is encoded by the small RNA segment 7 (S7). VP7 is extremely hydrophobic, the hydrophobic regions resemble those associated with the lipid-free protein coats of fd-type bacteriophages. Of the two major core proteins VP7 is the more abundant (Roy, 1992). VP7 comprises approximately one-third of the total protein content of the virion and is the main component of the capsomeres on the surface

of the core particle (Huisman & Van Dijk, 1990). The outer layer of AHSV has 260 knoblike capsomeres made up of trimers of VP7, each virion would then have 780 molecules of VP7. These protrusions are arranged in such a way that there are 132 channels at all five- and six-coordinated centers. It is likely that the network of aqueous channels provides pathways for the necessary metabolites to reach the transcriptional sites and for the nascent mRNA molecules to exit (Prasad *et al.*, 1992; Maree *et al.*, 1998b). There is also some evidence that the protein is not completely shielded by the outer capsid and can be recognised in the double-layered virus particle by VP7-specific antibodies (Huisman & Van Dijk, 1990). At least three antigenic sites have so far been mapped on BTV VP7, of which two appear to be linear determinants and one a conformational determinant. One linear determinant is located at the C terminus (amino acid residues 339 to 349), the other between residues 122 and 139, whereas the conformational determinant is located near the N terminus. VP7 is a group specific antigen (Chuma *et al.*, 1992). Disc-shaped aggregates of expressed AHSV-4 VP7 have been observed in recombinant baculovirus-infected insect cells. The flat, usually hexagonal crystals of VP7 produced by AHSV-9 infection of BHK-21 cells have a highly ordered hexagonal lattice consistent with a dimeric or trimeric subunit structure with a maximum diameter of 25  $\mu\text{m}$  and length of 250  $\mu\text{m}$ . The hexagonal arrangement observed in the VP7 crystal lattice appears to have direct structural similarity to the segmented, ring-shaped capsomeres that are visible on the outer surface of the AHSV core (Chuma *et al.*, 1992; Burroughs *et al.*, 1994).

### 1.5.2. VP3

The third largest RNA segment (L3) encodes the VP3 protein and it forms an inner shell that is the main structure of the subcore (Roy, 1992). The inner shell of each virus consists of 120 copies of the VP3 protein and can retain its structure even if the outer VP7 core layer is lost (Grimes *et al.*, 1998). This structure forms the protein scaffold on which the capsomeres and the three internal minor proteins, VP1, VP4 and VP6 are arranged (Huisman & Van Dijk, 1990; Maree *et al.*, 1998a). VP3 is hydrophobic in nature and plays an important role in the structural integrity of the virus core (Roy, 1992). VP3 molecules can also bind RNA and the smallest viral particles containing RNA are the VP3 subcores. The VP3 molecule therefore seems to play a fundamental role in the early stages of formation of the virus core (Grimes *et al.*, 1998).

VP2 in rotavirus is the equivalent of the VP3 in BTV (Prasad *et al.*, 1992). VP2 is an RNA-binding protein with nonspecific affinity for both ssRNA and dsRNA, but with a definite preference for ssRNA. The RNA-binding domain of VP2 is located near the N terminus of the protein. VP2 is essential for RNA replication as the VP1 protein requires the presence of VP2 for replicase activity (Boyle and Holmes, 1986; Patton & Chen, 1999). VP2 could be considered as the rotavirus nucleocapsid protein (Kumar *et al.*, 1989).

### 1.5.3. CO-EXPRESSION OF VP7 AND VP3

The baculovirus expression system has been used to co-express VP3 and VP7 to determine whether cores can be synthesised in the absence of the dsRNA species or other structural or non-structural proteins. For this purpose a dual expression plasmid vector has been made to receive the cDNA genes and to introduce them into a baculovirus. After infection of insect cells spherical particles were observed under the electron microscope. These particles were found to consist of empty core like particles (CLPs) whose size and appearance are similar to authentic cores prepared from BTV or authentic AHSV cores with a diameter of 72 nm. From the observed synthesis of empty CLPs by the expression of VP3 and VP7 proteins, it can be concluded that their formation is not dependent on anything else than the presence of these two proteins (Prasad *et al.*, 1992; Roy, 1992; Maree *et al.*, 1998b). Each icosahedral building block that makes up the core is composed of 13 molecules of VP7 attached to two molecules of VP3 so that a total of 60 subunits are formed (Grimes *et al.*, 1998). When expressed independently of VP3 in baculovirus, VP7 does not form icosahedral structures. It is therefore likely that VP7 requires close interaction with VP3 (Prasad *et al.*, 1992). It has been shown that expression of VP3, VP7 and VP1, results in the encapsidation of the VP1 protein *in vivo*. Similar data have been obtained for the encapsidation of the VP4 and VP6 proteins. When the VP2 and VP5 genes are co-expressed in the absence of the VP3 and VP7 core genes, the proteins fail to assemble (Liu *et al.*, 1992).

## 1.6. THE OUTER STRUCTURAL PROTEINS

These two proteins, VP2 and VP5, constitute approximately 40% of the total protein content of the virus. The fact that VP2 can be dissociated from the virion without removal of VP5 indicates that VP5 is more closely associated with the core particle than VP2. If VP5 has to recognise or bind both VP2 and one of the core proteins, it would place a higher degree of constraint on the variability of VP5 than on VP2 (Huismans & Van Dijk, 1990). VP5 or VP2 can bind to core-like particles (CLPs) independently of each other. VP2 and VP5 probably interact only with the VP7 protein of the core. However, since the level of interaction of VP2 and VP5 with CLPs is much greater when the proteins are both present than when they are added individually, it is likely that during the morphogenesis of the virus the proteins stabilize each other in the virion structure. The conserved regions of VP2 are probably necessary for maintaining the conformation of the molecule, as well as for interaction with the VP5 and VP7 proteins (Liu *et al.*, 1992).

### 1.6.1. VP2

VP2 is encoded by RNA segment L2. This protein is the most variable of all the viral proteins and is the main serotype specific antigen, VP2 is also the viral haemagglutinin (Fukusho *et al.*, 1989; Roy, 1989; Roy, 1992). Peptide mapping has indicated that VP2 is unique for each of the US BTV-serotypes (Huismans & Van Dijk, 1990). Antisera raised in rabbits following immunisation with baculovirus-expressed BTV-10 VP2 protein efficiently neutralise the infectivity of BTV-10. In addition, the antisera neutralise, to lesser extents, certain other BTV serotypes (BTV-20, -11, -17 and - 4), and antisera raised against VP2 proteins of other BTV serotypes also neutralise the respective homologous virus and cross-neutralise to lesser extents a number of other BTV-serotypes. This data indicate that some BTV serotypes are more closely related than others. The neutralising effect of VP2 also indicates its potential of using it as a subunit vaccine (Huismans *et al.*, 1987a; Huismans & Van Dijk, 1990; Roy *et al.*, 1990; Roy, 1992). The major neutralising epitopes for AHSV are also located on VP2 (Burrage *et al.*, 1993; Martinez-Torrecuadrada *et al.*, 1994). Purified VP2 protein appears to be a suitable candidate for the development of a diagnostic kit useful for (1) detection of AHSV-specific antibodies in field samples, (2) typing of the samples for precise identification of the circulating serotype, and (3) evaluation of the level of protecting antibodies (Martinez-Torrecuadrada *et al.*, 1994). There is some evidence that VP2 is involved in cellular attachment. A loss of infectivity is associated with the removal of VP2 and such particles can also no longer bind to cells. Intermediate subviral particles of BTV that contained VP2

chymotrypsin cleavage products were, however, fully infectious. Although cleavage of VP2 did not affect cell attachment, it did result in a loss of haemagglutination activity. This function is generally also associated with VP2 since haemagglutination inhibiting antibodies to BTV are type-specific. The results suggest that the sites for haemagglutination activity and cell attachment are not necessarily the same (Eaton *et al.*, 1990; Huismans & Van Dijk, 1990).

### **1.6.2. VP5**

VP5 is encoded by RNA segment M5. This protein is more variable than all the core proteins but less than VP2. Although the protein is located in the outer capsid it does not appear to have any distinct neutralising activity. Antisera raised against baculovirus-expressed VP5 protein do not demonstrate any neutralising activity *in vitro*. However in contrast to these results it was found that when sheep were vaccinated with a VP2-VP5 combination, they had a higher neutralising antibody response than sheep vaccinated with VP2 alone. Perhaps VP5 enhances the immune response indirectly by interaction with VP2, and by affecting the conformation of VP2 and, consequently, its serological properties (Roy, 1992).

### **1.6.3. CO-EXPRESSION OF VP2, VP3, VP5 AND VP7**

Co-expression of VP2, VP3, VP5 and VP7 in insect cells using dual expression vectors in the baculovirus expression system led to the assembly of double-shelled virus like particles (VLPs) containing all four major structural proteins. The assembly of all seven structural proteins of BTV into VLPs has also been demonstrated in the absence of the viral genome and the four non-structural proteins (Roy, 1992). It appears that the assembly of BTV proteins occurs in various stages. Probably an unstable icosahedral particle composed only of VP3 is synthesised first, which interacts immediately with the three minor proteins. This subcore then forms a scaffold for the attachment of VP7 trimers. Either before or immediately after the CLPs are formed, the 10 RNA species become encapsidated. Eventually the VP2 and VP5 proteins simultaneously interact with the core to form the outer capsid of the virus particles (Liu *et al.*, 1992; Belyaev & Roy, 1993). A recombinant baculovirus quadruple expression vector was also made that included all four genes and made virtually homogenous VLPs (Belyaev & Roy, 1993).



## 1.7. THE NONSTRUCTURAL PROTEINS

### 1.7.1 NS1

It has been observed that NS1 genes are transcribed at a significantly higher rate than the other orbivirus genome segments and that NS1 is also synthesized in a large excess over other proteins in infected cells (Nel & Huismans, 1991). The AHSV-6 NS1 gene was found to be 1748 nucleotides in length. The longest single open reading frame (ORF) contains 1645 nucleotides and begins with an AUG at position 36 to 38 and terminates at positions 1680 to 1682 with an UAA codon. The ORF of the NS1 gene of AHSV-6 encodes a polypeptide of 548 amino acids with an estimated molecular mass of 63 478 Da and a net charge of +5 at a neutral pH (Maree & Huismans, 1997). NS1 is rich in cysteine and all 16 cysteine residues are conserved in the NS1 proteins of the different BTV serotypes. This suggests that NS1 has a highly ordered disulfide-bonded structure. In addition NS1 possesses several very strong hydrophobic regions which may play a role, along with those regions that contain the half-cysteine residues, in the formation of tubules (Nel *et al.*, 1990; Grubman & Lewis, 1992; Roy, 1992).

Tubules are routinely observed in orbivirus infected cells (Roy, 1989). When the NS1 gene is expressed in insect cells by a recombinant baculovirus, tubules similar to those found in virus infected mammalian cells are formed, confirming that the tubules are multimeric forms of the NS1 protein only (Eaton *et al.*, 1988; Urakawa & Roy, 1988; Roy, 1989; Eaton *et al.*, 1990; Huismans & Van Dijk, 1990; Thomas *et al.*, 1990; Nel & Huismans, 1991; Roy, 1992). The BTV NS1 tubules have a diameter of 68 nm and are composed of a coiled ribbon of NS1 dimers with 22 dimers per helix turn. In contrast AHSV tubules have an average diameter of  $23 \pm 2$  nm, which vary in length up to 4  $\mu$ m. These AHSV tubules have an internal structure with a fine reticular "cross-weave" appearance, which is very different in appearance to that of BTV and EHDV tubules. There is no evidence of the ladder-like or segmented appearance found in the much wider BTV (68 nm) and EHDV (52 nm) tubules (Huismans & Els, 1979; Maree & Huismans, 1997; Van Staden *et al.*, 1998). The tubules occur in the cytoplasm as bundles with random orientations. Condensation of NS1 into tubules appears to occur very rapidly and the majority of NS1 synthesised in a 30 min period is only recovered in a particulate form (Nel & Huismans, 1991). These tubules are believed

to be attached to the intermediate filament components of the cytoskeleton of the cell and are presumed to be involved in some way in the virus replication or transportation process (Roy, 1989; Roy, 1992). NS1 tubules may play a role in transport of mature virus particles from the virus inclusion bodies to the cell membrane where NS3 is involved in virus release (Maree & Huismans, 1997).

### 1.7.2. NS2

The only virus-specific phosphoprotein that can be identified in orbivirus-infected cells is NS2, the product of the S8 gene (Fukusho *et al.*, 1989; Roy, 1989; Grubman & Lewis, 1992; Roy 1992). NS2 purified by affinity chromatography can also be phosphorylated *in vitro* without addition of exogenous added phosphokinase, suggesting that the kinase responsible for phosphorylation remains associated with NS2 during purification (Huismans & Van Dijk, 1990). This protein is very rich in charged amino acids (aspartate, glutamate and lysine residues) that are hydrophilic and that might interact with nucleic acids (Thomas *et al.*, 1990). Hall *et al.* (1989) was the first to sequence a NS2 gene, namely the NS2 gene of BTV serotype 10. The sequence is 1124 base pairs in length and contains a single open reading frame which codes for a protein of 357 amino acids. The protein has a molecular weight of 41153 Daltons and a net charge of -4 at pH 7.0. The C-terminal half of the protein is relatively hydrophilic and analysis of the amino acid sequence has revealed that NS2 does not contain the highly conserved sequence motifs which are characteristic of the catalytic domains of all known protein kinases. Therefore, it is unlikely that NS2 has kinase activity (Hall *et al.*, 1989).

Grubman *et al.* (1990) reported that segment 8 of BTV serotype 17 contains 1125 base pairs and codes for a protein of 40581 Daltons containing 354 amino acids with a net charge of -8.5 at pH 7.0. The carboxyl terminal portion of both BTV NS2 proteins have a high degree of potential  $\alpha$ -helix structure (Grubman *et al.*, 1990). Segment 8 of bluetongue virus serotype 1 from South Africa is 1124 bp in length, with an open reading frame of 357 amino acids. Sequence comparison with data already published for the equivalent genome segment of BTV serotype 10 gave homologies of 89.15% at the nucleotide level and 93.27% at the amino acid level (Wade-Evans, 1992). EHDV segment 8 is 1185 bp in length with 5' and 3' noncoding regions of 19 and 38 bp, respectively. The AHSV segment 8 gene is 1166 bp with the corresponding noncoding regions 22

and 49 bp in length. The 5' and 3' terminal hexanucleotide sequences of EHDV segment 8 are identical to the conserved terminal sequences of all known BTV and EHDV genome segments (5' GUUAAA and ACUUAC 3') while the AHSV segment 8 sequence differs from the consensus sequence by the substitution of a U for an A at the fourth position from the 5' end and an A for a U at the fourth last position from the 3' end. On the basis of similar variations observed in the terminal sequences of other AHSV cloned genome segments a consensus sequence of 5' GUUXAA (X = A, U, or C) and ACXUAC (X = A or U) 3' for AHSV was proposed. An additional feature that has previously been identified in other orbivirus genome segments, namely the presence of inverted repeats adjacent to the conserved terminal sequences was also found in EHDV and AHSV segment 8. This confirms the importance that the secondary structure of mRNA could have in events such as packaging of RNA. In general the amino acid sequences at the N-terminal half of NS2 are more conserved than the sequences at the C-terminal half, even across the different orbiviruses (Van Staden *et al.*, 1991).

NS2 is synthesised to a high level in infected cells, indicating its important role in the virus life cycle. It has been demonstrated that NS2 is present mostly in viral inclusion bodies (VIBs) and that it is not associated with virions (Roy, 1992; Uitenweerde *et al.*, 1995). Its presence within VIBs is consistent with a role for this protein in RNA synthesis or sequestration of viral single-stranded RNA molecules prior to virus morphogenesis. These VIBs have also been shown to contain dsRNA, virus-specific polypeptide and both complete and incomplete viral particles (Eaton *et al.*, 1990). These VIBs which are composed of a dense fibrillar network have been shown to react with monoclonal and monospecific antibodies to the non-structural protein NS2 (Roy, 1989). Thomas *et al.* (1990) have also presented evidence that when NS2 protein is synthesised independently of any other BTV-encoded proteins the inclusion bodies are still formed and are structurally very similar to the VIBs of BTV. This is direct proof that the major-nonstructural protein NS2 is indeed responsible for formation of the large inclusion bodies.

NS2 protein has been shown to be capable of binding single stranded RNA (Roy, 1989; Thomas *et al.*, 1990) and is thought to be subsequently involved in the selection and condensation of the mRNA segments into precursor viral particles (Uitenweerde *et al.*, 1995). The protein exists predominantly in a multimeric form. *In vitro* studies with baculovirus-expressed NS2 have shown that NS2 is phosphorylated at specific serine residues (amino acids 185 and 308 in particular, but

also residues 182, 289, 291, and 292). To what extent these sites are phosphorylated in the population of NS2 molecules is unknown. The cellular enzyme(s) responsible for the phosphorylation is also not known. In an attempt to determine whether phosphorylation is important in RNA binding, studies have shown that dephosphorylated NS2 protein binds ssRNA efficiently. Thus phosphorylation does not appear to be important in RNA binding (Roy, 1992).

An analysis of the potential secondary structure of NS2 protein indicated that it is probably abundant in  $\beta$  plates, especially at the N-terminus, while the C-terminus is  $\alpha$ -helix-rich (Roy, 1989; Uitenweerde *et al.*, 1995). NS2 was detected in both the soluble and particulate fractions of infected cells. Particulate NS2 can be solubilised by high-salt treatment and can then be purified using affinity chromatography. It was found that mixtures of BTV mRNA and soluble NS2 form a complex with an estimated S value of about 22 on sucrose gradients. The S value was independent of the mRNA/NS2 ratio. An attractive hypothesis for the function of NS2 is that it acts in the selection and condensation of the ten mRNA species during virus morphogenesis (Huismans & Van Dijk, 1990). In one study, deletion of up to 130 amino acids from the C-terminus of BTV NS2 did not abolish the RNA-binding activity. In contrast, the removal of even 40 amino acids from the N-terminus totally prevents ssRNA binding. These data indicate that the N- but not the C-terminal domain of NS2 is needed for protein-RNA interaction (Roy, 1992).

### 1.7.3. NS3 AND NS3A

The two smallest non-structural proteins, NS3 and NS3A, are synthesised in low abundance in infected cells and both proteins are encoded by segment 10 (S10). From sequence analyses NS3 and NS3A are estimated to be approximately 25.5 and 24 kDa, respectively. (French *et al.*, 1989; Huismans & Van Dijk, 1990; Roy 1992; Wu *et al.*, 1992; Sailleau *et al.*, 1997). The S10 gene has two conserved in-frame methionine/AUG translation initiation codons. NS3 and NS3A are derived from alternative translation initiation sites so that they differ only with respect to the ten additional amino acids present at the N terminus of NS3. Deletion of the first AUG abolishes synthesis of NS3 but not that of NS3A (Van Dijk & Huismans, 1988; Van Staden & Huismans, 1991; Roy, 1992; Van Staden *et al.*, 1998). The first initiation codon is located at nucleotide positions 20-22 in all AHSV serotypes so far analysed, except AHSV-9 in which it is at nucleotide 19-21 as in Palyam virus. The second initiation codon is slightly more variable, being at position 50-52 in

AHSV-1, 3 and 8, at nucleotide 52-54 in AHSV-9 and Palyam virus, and at 53-55 in AHSV-4 (De Sá *et al.*, 1994).

Predicted protein sequence analyses of the encoded products of BTV-10 S10 dsRNA have revealed at least two conserved hydrophobic domains (amino acid residues 118 to 147 and 156 to 182 respectively), which may serve as transmembrane domains. In addition, two potential glycosylation sites (amino acid residues 63 to 65 and 150 to 152) are also present. It has also been reported that NS3 and NS3A are associated with intracellular, smooth-surfaced vesicles and the plasma membrane. This suggests that the proteins may be involved in the final stages of virus morphogenesis, namely the release of the virus from infected cells. NS3 may share some functional similarities with the rotavirus glycoprotein NSP4, which mediates the binding of rotavirus particles to the rough endoplasmic reticulum and the acquisition of a transient envelope. Data have been obtained which indicate that NS3 and NS3A containing *N*-linked high-mannose sugars are transported to the Golgi apparatus, after maturation in the ER, and modified into *N*-linked complex glycoproteins. Apparently these are further converted into lactosaminoglycan proteins. Almost all proteins containing poly-lactosaminoglycan are membrane bound. The proteins are then transported onto the cell plasma membrane. It has been suggested that *N*-linked glycosylation might play a role in conferring transport competence to some integral membrane proteins and that this posttranslational modification might in some instance provide a signal for transport out of the ER. However, addition of tunicamycin or cycloheximide did not prevent the transportation of NS3 and NS3A proteins to the Golgi complex. Therefore, *N*-linked glycosylation of the NS3 gene products is not required for their intracellular transport (Roy, 1992; Wu *et al.*, 1992; Van Staden *et al.*, 1995). Mutation studies involving the two potential membrane-spanning and the glycosylation site at amino acid 150 between them in BTV NS3 indicated that both the amino- and carboxy-terminal ends of the protein are cytoplasmic (Bansal *et al.*, 1998).

Virus-like particles (VLPs) synthesised by recombinant baculoviruses are associated with the cytoskeleton of the insect cytoplasm. However such an association is disrupted and these particles are secreted by budding through the cellular membrane if the insect cells are co-infected simultaneously with a recombinant baculovirus expressing the S10 gene products along with the two dual recombinant viruses that are capable of VLP formation. These findings indicate a role for NS3 proteins in the release of virus from the infected cells (Roy, 1992; Wu *et al.*, 1992; Jensen

*et al.*, 1994; Van Staden *et al.*, 1995).

The rotavirus nonstructural protein NSP4, which has been proposed to share functional similarities with NS3, is 175 amino acids long and possesses an uncleaved signal sequence and two amino-terminal glycosylation sites. Utilising one of three potential hydrophobic domains, the protein spans the membrane only once, with the glycosylated amino-terminal region oriented to the luminal side of the ER and the carboxy-terminal region to the cytoplasmic side (Bergman *et al.*, 1989; Sailleau *et al.*, 1997). NSP4 acts as a receptor that binds single-shelled rotavirus particles and mediates their budding into the lumen of the rough endoplasmic reticulum during viral maturation. The cytoplasmic tail of NSP4 seems to be necessary for this receptor activity. Similarly, the N-terminal region of the orbivirus NS3 proteins could mediate the recognition or interaction of NS3 with viral components (Mattion *et al.*, 1992; Sailleau *et al.*, 1997).

The proposed orientation of NS3 is with the N- and C-termini on the cytoplasmic side of the membrane, with each of the two potential transmembrane regions spanning the membrane. A novel feature of NS3 was that its expression appeared to have a cytotoxic effect on the host cells. Five different domains which were thought to be possible structural or catalytic sites of the AHSV NS3 protein, were identified for modification. These include two hydrophobic domains (TM1 and TM2: amino acids 226-137 and amino acids 154-176) that are predicted to form transmembrane helical domains; a cluster of conserved proline residues (amino acids 22-34); an amino acid region (amino acids 43-92) showing very strong conservation between all AHSV NS3 proteins, as well as among other orbiviruses; and the amino terminus of the NS3 protein (to force expression of only NS3A by selective deletion of the 5' end of the gene). Substitution of four amino acids in either the first or the second transmembrane region was sufficient to abolish or diminish the cytotoxic effect. It is thought likely that this was due to an alteration in the structure of the protein which rendered it incapable of interacting with cellular membrane components (Van Staden *et al.*, 1995; Van Staden *et al.*, 1998; Van Niekerk, personal communication).

## 1.8. PROPERTIES AND POSSIBLE FUNCTIONS OF THE NONSTRUCTURAL PROTEIN NS2

Now that we had a brief view of AHSV in general, the rest of this chapter will focus on the properties and possible functions of the nonstructural protein NS2 of AHSV. First we will look at NS2's ssRNA binding ability as well as a few other proteins of different viruses and their ability to bind ssRNA. As ssRNA binding is one of NS2's most known properties it probably plays an important role in its function in the virus. Secondly we will look at phosphorylation as a property of NS2. One of the possible functions assigned to NS2 is that it may play a role in encapsidation with the selection and condensation of the mRNA segments into precursor viral particles. The last subject that will be focussed on will then be virus encapsidation. If we understand virus encapsidation we might get a better idea of how NS2 may play a role in the process.

### 1.8.1. SINGLE-STRANDED RNA-BINDING ABILITY

According to Lee *et al.* (1993) two classes of prokaryotic and eukaryotic RNA-binding proteins have been identified. One contains a RNA recognition motif, which consists of 80 amino acids with two conserved sequences, RNP-1 and RNP-2, of 8 and 6 amino acids, respectively. This class of RNA-binding proteins includes proteins which bind to pre-mRNA, mRNA, small nuclear RNA, and pre-rRNA and are involved in the regulation of translation, splicing, and a variety of other activities. The other class contains an arginine-rich motif (ARM) with a core of 4 to 8 amino acids, mostly arginines. The ARM sequence itself is sufficient for specific RNA recognition. The *tat* and *rev* proteins of human immunodeficiency virus type 1 (HIV-1), the N protein of bacteriophage lambda, and the capsid proteins of some RNA viruses belong to this family.

The NS2 protein of AHSV and BTV binds to ssRNA, as is the case for the nonstructural protein NS3 of reovirus and NSP3 of rotavirus. Reoviruses share with rotaviruses the ability to form reassortant viruses at high frequency when cells are simultaneously infected with two different strains of virus. This ability indicates that there must be a stage in viral infection when plus-stranded segments are able to assort freely prior to the incorporation of an appropriate selection of 10 segments into the progeny particle (Bellamy & Both, 1990). Hydrodynamic studies of the flexibility or stiffness of isolated rotavirus RNA segments in solution have indicated that the RNA segments cannot be packaged into the rotavirus capsid unless intimate protein-RNA interactions

take place. In solution the RNA molecules possess a 'wormlike' or flexible cylinder structure; as example, RNA segment 1 of rotaviruses (3,302 base pairs and a contour length of 928 nm) theoretically can not bend into a capsid of 50 nm as a free molecule because the persistence length is 112.5 nm. Therefore, to obtain RNA flexibility, one has to assume that intimate protein-RNA interaction occur in the virion to induce the needed bending and packaging of the dsRNA segments into the virus capsid (Estes & Cohen, 1989). Some highly specific mechanism must be responsible for assembling 10 discrete species of RNA into each virus particle. This mechanism must be very efficient, since under optimal conditions the ratio of reovirus particles to infectious units has been reported to approach unity. Although this mechanism is efficient (since most virus particles contain 10 genes), it is not infallible, and several examples of reovirus particles with less than 10 genes are known (Joklik, 1981).

Antczak & Joklik (1992) demonstrated that reovirus mRNA's associate with proteins very soon after they are transcribed. Three reovirus-specified proteins are primarily involved: the two nonstructural proteins  $\mu$ NS and  $\sigma$ NS, and protein  $\sigma$ 3. Association with  $\mu$ NS was generally the interaction that occurred first, followed shortly by association with  $\sigma$ NS and  $\sigma$ 3, and during the most active phase of reovirus mRNA synthesis, these three proteins were associated with ssRNA to a roughly equal extent. These ssRNA-containing complexes contain ten to thirty protein molecules, which means that the complexes contain about 40% RNA. Ten to thirty proteins can easily be accommodated in ssRNA-containing complexes because protein-binding sites in RNAs are unlikely to exceed 40 nucleotides, which implies that substantially less than one-half of the sequences of ssRNAs would be in close contact with proteins. Proteins  $\mu$ NS and  $\sigma$ 3 do not contain conserved RNA-binding motifs that are present in other proteins. Protein  $\sigma$ NS is the counterpart of the orbivirus NS2 protein and contains, like it, the 9 amino acid long sequence (I/L)XXM (I/L) (S/T)XXG, this sequence was proposed to play a role in RNA binding (Gomatos *et al.*, 1981; Van Staden *et al.*, 1991; Antczak & Joklik, 1992). This consensus sequence is also present in rotavirus protein NSP3 between amino acid 104 and 112 in the basic domain (Mattion *et al.*, 1992; Poncet *et al.*, 1993).

According to Eaton *et al.* (1990) the nonstructural protein  $\sigma$ NS may play a role in the process whereby ten individual single-stranded parental RNA transcripts are sequestered into a precursor particle. It is known that  $\sigma$ NS plays an important role early in the reovirus replication cycle, as a



temperature-sensitive mutant defective in  $\sigma$ NS protein was unable to synthesise dsRNA at the restrictive temperature. (Richardson & Furuichi, 1985; Thomas *et al.*, 1990). It has been shown that  $\sigma$ NS, encoded by the mammalian reovirus (MRV) S3 gene, as well as  $\sigma$ NS, encoded by the S4 gene of avian reovirus (ARV), bind to reovirus single-stranded RNA and may play an important role in the earliest stages of particle assembly. Competition assays showed that ssRNA from an unrelated avian pathogen infectious bursal disease virus was able to compete for binding of  $\sigma$ NS to poly(A)-Sepharose. These data suggest that ARV  $\sigma$ NS binds to ssRNA in a nucleotide sequence non-specific manner and is functionally similar to its counterpart specified by mammalian reovirus.  $\sigma$ NS did not bind to dsRNA or DNA (Poncet *et al.*, 1993; Yin & Lee, 1998).

Studies on the cognate nonstructural protein NSP3 of rotavirus indicated that NSP3-bound RNAs contained sequences included in each of the 11 rotavirus mRNAs. All of the RNAs showed the presence of the same sequence, ...UGUGAXX, at their 3' ends. This sequence matched the 3' end conserved sequence of rotavirus RNAs. The nonstructural viral protein NSP3 is bound to the 3' end of rotavirus mRNA and protects the whole 3' end consensus sequence from RNase T1, digestion. The mRNAs linked to NSP3 correspond to the 11 rotavirus genes (Poncet *et al.*, 1993). Another nonstructural protein of rotavirus, NSP1, also possesses nonspecific RNA-binding activity. It is encoded by genome segment 8 of SA11 rotavirus and exists in high concentrations in the infected cell where it localises to the viroplasm. Analysis of the growth characteristics of tsE, a SA11 temperature-sensitive mutant that maps to segment 8, indicates that NSP1 is essential both for the formation of the viroplasm and for genome replication. At nonpermissive temperature, empty particles accumulate in tsE-infected cells suggesting that NSP1 is important for the packaging of viral RNA (Patton *et al.*, 1993). It was found that NSP1, as its levels increase during infection, associates with newly synthesized viral mRNAs and directs the transcripts to the viroplasm (Kattoura *et al.*, 1992). Comparison of the predicted sequences of NSP1 showed that all possessed a conserved basic domain of 37 amino acids at residues 205-241 that was extremely basic in nature (pH 11.5-12) and thus may serve as the RNA-binding domain (Patton *et al.*, 1993).

BTV NS2 has been shown to bind single-stranded RNA and have the highest affinity for the 3' regions of the mRNA (Eaton *et al.*, 1990; Theron & Nel, 1997). Mutant BTV transcripts lacking the

highly conserved 5'- and 3'-terminal hexanucleotides however indicated that these sequences are not necessary for optimal binding to NS2. Theron & Nel (1997) further characterised the NS2-RNA interaction by competition experiments between labelled BTV S8-RNA and homologous and heterologous RNAs. Although the various ssRNA species competed for binding to NS2, quite different binding affinities were observed at low molar excesses of the competitor RNAs. The BTV NS2 protein appeared to interact preferentially with BTV S8-RNA. Rabies virus RNA competed very inefficiently for binding to BTV NS2, while a level of interaction was detected with EHDV S8-RNA. Thomas *et al.* (1990) have also demonstrated that the NS2 protein not only has a strong affinity for BTV transcripts but also binds non-specifically to other ssRNA species indicating that the binding motif of the ssRNA is non-specific.

Both rotavirus NSP3 and reovirus  $\sigma$ NS have been shown to multimerise as smaller subunits that can assemble to form larger ones. The multimers are stabilised by binding to the 3' ends of viral mRNA (Huismans & Joklik, 1976; Mattion *et al.*, 1992; Uitenweerde *et al.*, 1995). Prior to RNase A treatment, the  $\sigma$ NS in cell extracts is found in large complexes ( $\geq 30$  S) that contain RNA. Once the complexes are treated with RNase A, they migrate between 13 and 19 S. Although RNase A treatment appears to remove much of the RNA it is hypothesised that small RNA fragments remain bound in the 13-19 S complexes. These RNAs may be protected from RNase A cleavage by binding to  $\sigma$ NS and/or may be small purine-rich regions that are insensitive to RNase A. The 7-9 S complexes contain only  $\sigma$ NS. They can be unmasked in several ways: exposing 13-19 S complexes to high salt, treating 13-19 S complexes with thermolysin, or expressing  $\sigma$ NS as a N-terminal deletion (Gillian & Nibert, 1998).

Evidence is presented that NS2 of both AHSV and BTV also multimerise by means of intermolecular and intramolecular disulfide bonds in the form of a protein with a sedimentation value of approximately 7S in both the orbivirus and baculovirus expression systems. Each multimer appears to be composed of six or more covalently bound monomers. Although many factors other than molecular mass influence the sedimentation of a protein, this result was corroborated by the sedimentation of the 7S multimer in the region of gradients where proteins of MW  $\geq 200$  kDa sediment. This 7S NS2 multimer binds ssRNA *in vitro* (Uitenweerde *et al.*, 1995).

Zhao *et al.* (1994) made a number of truncated BTV NS2 proteins, as well as single amino acid

mutations. The deletion derivatives and point mutants were used in ssRNA binding experiments. The data indicate that the carboxy terminus of NS2 (including the last 130 amino acids) is not required for ssRNA binding or for oligomerisation. While the amino terminus of NS2 (up to residue 92) was also found not to be required for oligomerization, certain amino acids located at the amino terminus, i.e., the arginines at residues 6 and 7 and a lysine at residue 4, but not a glutamic acid at residue 2, were required for ssRNA binding. Mutation of the lysine at residue 4 totally abrogated ssRNA binding, while mutation of the arginine at residue 6 or 7 led to significantly reduced binding of NS2 to ssRNA. The data demonstrated that the two activities of the protein are not associated with each other (Zhao *et al.*, 1994). The N-terminal eight amino acids of NS2 are required for RNA binding, but not for forming inclusion bodies. Data obtained by both limited proteolysis and deletion mutagenesis of reovirus  $\sigma$ NS, indicate that amino acids near the N terminus are also important for both binding RNA and forming or maintaining complexes larger than 7-9 S. Other data obtained using  $\sigma$ NS C-terminal truncations from *in vitro* transcription and translation, provide evidence that sequences within the N-terminal 118 amino acids of  $\sigma$ NS are sufficient for RNA binding (Richardson & Furuichi, 1983).

Tomato spotted wilt virus (TSWV) N protein binds to single-stranded RNA but not double-stranded RNA and is not sequence-specific in its binding to single-strand RNA *in vitro*, just like AHSV NS2. N protein has also been found to regulate its own synthesis through the encapsidation of the N mRNA, suggesting that *in vitro* N protein encapsidates the input single-stranded RNA (Richmond *et al.*, 1998).

The movement protein of tobacco mosaic virus (Osman *et al.*, 1993) is a ssRNA binding protein. Movement proteins facilitate the cell to cell movement of infectious entities consisting of viral RNA associated with movement proteins. The  $\beta$ b protein of barley stripe mosaic virus (BSMV) is also a movement protein with ssRNA binding ability (Donald *et al.*, 1997). It was shown for carnation mottle carmovirus (CarMV) that its movement protein, p7, binds to ssRNA with an  $\alpha$ -helix domain. This indicates that  $\alpha$ -helices may play a major role in ssRNA recognition in movement proteins (Marcos, *et al.*, 1999). Influenza A matrix protein (M1) binds nonspecifically to RNA (Wakefield & Brownlee, 1989). M1 binds to RNA via two sequences, amino acids 90 to 108, containing a nuclear localization domain (RKLKR), and amino acids 135 to 165, containing a zinc finger motif (Ye *et al.*, 1999). Influenza A virus protein NS2 has also been shown to bind ssRNA in a

sequence-independent manner (Richardson & Akkina, 1991; Galaza *et al.*, 1992). NS2 also mediates the nuclear export of viral ribonucleoproteins (O'Neill *et al.*, 1998). Overexpression of the NS2 protein reduce the level of transcription of the virus RNA (Gómez-Puertas *et al.*, 1999).

### 1.8.2 PHOSPHORYLATION

NS2 protein of AHSV and BTV is just like NS26 of rotaviruses a phosphoprotein. The serine residues are phosphorylated (Devaney *et al.*, 1988; Bellamy & Both, 1990; Eaton *et al.*, 1990). In 1983 Richardson & Furuichi suggested that a necessary requirement for a virus assembly protein is the dissociation of protein and RNA before packaging. This could be accomplished via phosphorylation of these serine residues and neutralisation of the positive charges of basic amino acids. Alternatively, conversion of ssRNA to dsRNA would result in the release of protein from RNAs.

It was found that the HPLC elution profiles of the <sup>32</sup>P-labelled tryptic peptides of BTV and AHSV NS2 were very similar, possibly reflecting the presence of conserved phosphorylation sites on the NS2 proteins. NS2 of BTV-10 (SA) and AHSV-9 contain 21 and 28 serine residues, respectively. An alignment comparison of identical amino acids indicated that only 10 of these residues were in conserved positions in the two proteins (Grubman *et al.*, 1990; Van Staden *et al.*, 1991).

It was found that phosphorylation played no role in NS2s ability to bind to ssRNA. Only the serine residues in the NS2 molecule are phosphorylated and there are a total of 20 serine residues in each molecule (Thomas *et al.*, 1990). In contrast with Thomas *et al.* (1990), Theron *et al.* (1994) found that phosphorylation of NS2 plays an important regulatory role in the replication of orbiviruses as a clear correlation between phosphorylation of NS2 and a corresponding decrease in ssRNA-binding has been demonstrated *in vitro*. NS2 expressed in *E. coli* as a GST fusion protein was found not to be phosphorylated. Throughout a wide range of different ionic strengths, the phosphorylated protein preparations were found to be 20 to 30% less effective in binding ssRNA, compared to the unphosphorylated control preparations. In addition, phosphorylated NS2 appeared to be more sensitive to increased ionic strength as the RNA-binding capability of phosphorylated NS2 was completely abolished at 0.2M STE-TX, whereas more than 20% of the unphosphorylated NS2 could still bind RNA. NS2 is specifically phosphorylated by a cellular

kinase, as the unphosphorylated bacterial-expressed NS2 was phosphorylated when it was mixed with a cytoplasmic extract prepared from uninfected insect cells. In addition, it was found that the kinase associated with the cytoplasm of *S. frugiperda* cells exhibited a specificity for NS2, since the GST affinity tail was found to be unphosphorylated in control experiments. It appears likely that a single protein kinase is involved since it has been demonstrated that sucrose gradient purification or even poly(U)-Sepharose column purification of NS2 did not change its ability to be phosphorylated by the simple addition of [ $\gamma$ - $^{32}$ P]ATP, and it therefore follows that the cellular kinase was co-purified with the NS2 protein (Theron *et al.*, 1994; Uitenweerde *et al.*, 1995).

The hepatitis C virus NS5A protein is also a phosphoprotein that is phosphorylated on its serine residues. For this protein it was also shown that it is associated with, and is phosphorylated by a cellular protein kinase (Ide *et al.*, 1997). It is thus not uncommon for viral proteins to make use of cellular protein kinases in order to be phosphorylated.

Replication of rotavirus RNA is thought to occur in specialised regions of the cells called viroplasm where structural proteins (VP2 and VP6) as well as nonstructural proteins (NSP2 and NSP5) are concentrated. NSP5 is a phosphoprotein that has a high serine and threonine content. Sequence similarity with the family guanine kinases found at the C-terminal end of NSP5 and a putative nucleoside triphosphate binding site at the N-terminus of the protein support the hypothesis that NSP5 is a protein kinase. NSP5 could also be a RNA binding protein. Full phosphorylation of NSP5 which leads to multiple forms of the protein occurs in two steps. A first step of phosphorylation occurs after the synthesis of the 26 K protein and is the result of the intrinsic kinase activity of NSP5. The second step of phosphorylation is observed only when NSP5 is expressed together with all the other viral proteins and possibly after an alteration of the cell physiology induced by rotavirus infection and leads to the formation of the more heavily and multiple phosphorylated forms of NSP5 (Poncet *et al.*, 1997). NSP5 hyperphosphorylation can take place when NSP5 is co-expressed with NSP2. NSP2 also binds to ssRNA and there is also interaction between NSP2, NSP5 and the viral polymerase VP1 (Afrikanova *et al.*, 1998). NSP2 forms multimers and have an associated nonspecific NTPase activity. NSP2 is also phosphorylated and may function as a molecular motor by binding viral mRNA and catalysing its packaging through the energy generated by its NTPase activity (Taraporewala *et al.*, 1999).

### 1.8.3. ENCAPSIDATION

Encapsidation is an important event in the virus life cycle. In addition to providing the virus nucleic acid genome with a protective protein coat against nuclease degradation, it regulates many facets of viral biosynthesis (Duggal & Hall, 1993). In the case of dsRNA viruses the ssRNA species are encapsidated, with the minus strands subsequently being made using the plus strands as a template. It is assumed that only one of each of the segments is selectively encapsidated and replicated (Mattion *et al.*, 1992). Multiple RNA-protein interactions must occur in the processes of replication and packaging of an RNA virus (Ribas *et al.*, 1994a). In packaging, a RNA virus must pick out its genome from a sea of cellular mRNAs. This key process requires that a specific site(s) on the viral RNA be recognised by a viral protein(s). All dsRNA viruses package the viral (+) single-stranded RNA, converting it in the virus particle (or virus precursor particle) to the double-stranded form (Fujimura & Wickner, 1988; Icho & Wickner, 1989; Pattnaik *et al.*, 1992; Ribas *et al.*, 1994b). As the encapsidation process of AHSV is not known yet, I will discuss the encapsidation processes of other dsRNA viruses. These viruses may give us an idea of how AHSV encapsidates its RNA as well as give us a guideline as how to start unraveling the mystery.

The only dsRNA virus whose RNA packaging site has been determined is the L-A dsRNA virus of *Saccharomyces cerevisiae* which has a genome consisting of a single 4,6kb segment. L-A's *cis*-acting packaging signal, a 24-nucleotide stem-loop structure with a protruding A residue, is located 400 nucleotides from the 3' end of the viral positive strand (Shen & Bruenn, 1993; Ribas *et al.*, 1994a). The viral positive-strand ssRNA has two open reading frames (ORFs): ORF1, the 5' *gag* encoding the major coat protein (Gag) and ORF2, the 3' *pol* expressed only as a Gag-Pol fusion protein (180 kd) formed by a -1 ribosomal frameshift event in the region of overlap of *gag* and *pol* and is found in only about one molecule/virus particle (Icho & Wickner, 1989; Shen & Bruenn, 1993).

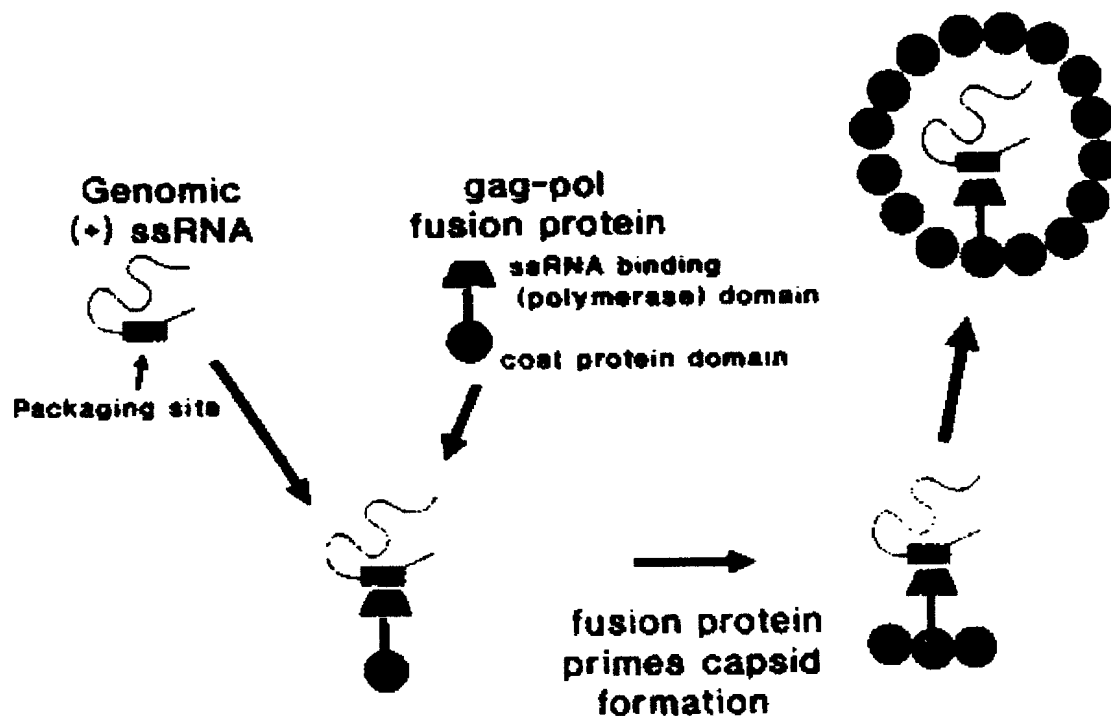
Pol is multifunctional protein with a N-terminal domain necessary for packaging of viral positive strands, amino acid sequence patterns typical of RNA-dependent RNA polymerases, and *in vitro* ssRNA binding activity (Ribas *et al.*, 1994a). Gag alone is sufficient to form morphologically intact (albeit, empty) virus particles, but it fails to package the viral single-stranded RNA genome (Fujimura *et al.*, 1992).

*In vitro* blotting experiments with fragments of Pol expressed in *E. coli* have already defined two RNA binding domains. The first is the N-terminal *in vitro* binding domain (residues 172 to 190) is located within the packaging domain and necessary for M1 propagation and for *in vivo* packaging. This sequence is IRPKHFKGLRLYTRSKVTA (Ribas *et al.*, 1994a; Ribas *et al.*, 1994b). The second is the *in vivo* packaging domain, Pol residues 67 to 213, that is much larger than the *in vitro* binding domain and are necessary for encapsidation of transcripts carrying the packaging signal. The deletion of just 5 residues (SPRRK) eliminates *in vivo* packaging, but not *in vitro* RNA binding (Ribas *et al.*, 1994b). The N-terminus also consists of a major coat protein monomer (Fujimura & Wickner, 1988).

The C-terminal RNA binding domain, encoded by the 3' reading frame (ORF2, residues 770 to 819) is not essential for packaging but is required for M1 propagation. M1 is a 1.8-kb dsRNA satellite of the L-A virus that needs the L-A proteins for its propagation (Ribas *et al.*, 1994a). ORF2 encodes a protein with a basic region that was shown to be highly  $\alpha$ -helical (Icho & Wickner, 1989). The C-terminal domain also contains a sequence pattern diagnostic of viral RNA-dependent RNA polymerases (Fujimura *et al.*, 1990). ORF1 (residues 573-590) shows some local similarity with the  $\sigma 3$  major virion structural protein (residues 129-148) of reovirus (Icho & Wickner, 1989). Most RNA-binding proteins utilise a combination of sequence and complex three-dimensional shapes to provide binding specificity (Finerty & Bass, 1997).

The RNA packaging domain of the Pol region of the L-A dsRNA virus Gag–Pol fusion protein first recognises the RNA packaging signal, located about 400 nucleotides from the 3' end of the positive strands. At this stage, negative-strand synthesis to form dsRNA is probably prevented until the Gag region of the Gag–Pol fusion protein has associated with free Gag protein to enclose the positive strands in the virus particle. Then, the RNA polymerase interacts with the internal replication enhancer, overlapping with the packaging signal and the 3'-end replication initiation site, and begins to synthesise negative strands to form dsRNA. In synthesizing negative strands, Pol must presumably release the packaging signal. Once dsRNA has been formed, the enzyme initiates transcription to make the viral positive strands which are extruded to act as mRNA and to be packaged in new particles (Fujimura *et al.*, 1992; Ribas *et al.*, 1994a). It was suggested that the C-terminal “*pol*” domain of the 180 kd “*gag-pol*”-like fusion protein binds viral (+) ssRNA, and the N-terminal “*gag*” domain then primes polymerisation of major coat protein to form the capsid,

thus ensuring the packaging of both the viral genome and the RNA polymerase. This model is supported by the fact that the ORF2 protein has ssRNA binding activity and serves to explain why L-A makes a *gag-pol*-like fusion protein (Fujimura *et al.*, 1990). The 180 kd protein's ssRNA binding (C-terminal) domain binds specific to viral (+) ssRNA and this binding initiates encapsidation. Then, the host factor becomes associated with the complex to provide all of the components for (-) strand synthesis. Next, the major coat protein (N-terminal) domain of the 180 kd protein may serve to prime capsid assembly by its homologous association with other coat protein monomers. The 180 kd protein and host factor in the closed particle now make (-) strands to form dsRNA and then (+) strands that are extruded from the particle to begin the cycle again (Fujimura & Wickner, 1988). Empty viral particles could: first, specifically bind to viral (+) strands to form stable RNA-protein complexes; second, perform RNA replication [(-) strand synthesis on an added (+) strand template] in the presence of a host factor and third, carry out RNA transcription [(+) strand synthesis on an added viral dsRNA template] (Fujimura *et al.*, 1990).



**Fig. 1.1.** L-A encapsidation model (Fujimura *et al.*, 1992)



There is proof of specific encapsidation signals in poliovirus RNA (Barclay *et al.*, 1998). The binding sequence on the X (+) strand was located between bases 131 and 154 and a stem-loop structure was predicted in this sequence. When either side of the stem sequence was substituted with the other side of the stem sequence, the modified RNA lost its binding activity to empty particles. However, changing both sides of the stem restores the stem structure and concomitantly restores the binding activity in the modified RNA. These results confirm the presence and importance of the stem in the binding sequence and indicate that while the stem-loop structure is necessary for binding, the sequence of the stem is not important (Fujimura *et al.*, 1990). According to Shen & Bruenn, (1993) the requirements for binding of viral particles to viral plus strands are: a first stem of 4-6 base pairs, a bulge loop of 1-4 bases, a short stem of three base pairs, and a loop with the sequence GAUYC. Sequence specificity seems to reside solely in the GAUYC-loop. Only 44 nucleotides, including X's binding site, are sufficient for encapsidation into L-A viral particles (Fujimura *et al.*, 1990).

Several of the five rotavirus nonstructural proteins have ssRNA binding activity. NSP3 protein has ssRNA binding activity that is sequence nonspecific *in vitro*, but *in vivo* cross-linking experiments show that it is specifically associated with the conserved 3' ends of viral (+) ssRNA. NSP1 has both ssRNA and dsRNA binding activity, but again has no sequence specificity *in vitro*. *In vivo*, NSP1 is associated with viral mRNA in the cytoplasmic region where viral replication is proceeding, the viroplasm. Mutants in this protein produce empty particles at the non-permissive temperature, suggesting that it has a role in packaging of viral (+) strands. The NSP1 protein has ssRNA binding activity with specific affinity *in vitro* for the 5' part of viral (+) strands (Ribas *et al.*, 1994b). NS2 of AHSV also has ssRNA binding activity (Uitenweerde *et al.*, 1995). Encapsidation of AHSV and the other dsRNA viruses may occur by the same model as the L-A virus. The only difference may be that where L-A uses only one protein for all the functions the other viruses may use a lot of different proteins that work together to the same end.

Animal dsRNA viruses, such as reovirus and rotavirus, contain a set of 10 or more dsRNA genome segments in each virion. Because the individual genome segments can be simultaneously transcribed in virions, a minimum of one polymerase molecule per genome segment must be present in the virion. The  $\lambda 3$  protein of reovirus is the RNA polymerase or a subunit thereof, and 12 or less  $\lambda 3$  molecules are present per virion; thus, there is about one RNA polymerase molecule

per packaged segment. Like the L-A virus, (+) ssRNA transcripts are the species packaged.  $\lambda 3$  is not made as a fusion protein, but this protein may specifically bind the genomic ssRNA segments and then, by interacting with other viral particle proteins, these RNA polymerase molecules could participate in the stoichiometric packaging of the (+) strands (Fujimura *et al.*, 1990).

Loudon & Roy (1992) showed that BTV core-like particles (CLPS) exhibited a nonspecific ability to bind nucleic acid. In nitrocellulose binding assays an equal affinity for BTV-specific and nonviral ssRNA was observed. Binding of dsDNA to the CLP was also demonstrated using sucrose gradient sedimentation. Using individually purified VP7 and VP3 in the form of subcores, the ability of the CLP to bind nucleic acids was shown to reside on VP3. The binding activity found on BTV VP3 has strong similarities with that of rotavirus VP2. Both bind ssRNA in a non-sequence-specific manner, and both also bind dsDNA. Rotavirus VP2 and BTV VP3 have other similarities. Both of these proteins are approximately 103 kDa in size and are predicted to have low negative charge at neutral pH. Both form a subviral particle, designated the core in rotavirus and the subcore in BTV. The function of an RNA binding domain on BTV VP3 may be involved in assembly. Studies with other members of the *Reoviridae* have shown that during assembly positive-sense ssRNA interacts with subviral particles. In rotaviruses, RNase-sensitive ssRNA molecules have been found extending from the surface of the subviral replicative intermediates. The RNA subsequently moves into the particle and is replicated while the remaining structural proteins assemble to form the single-shelled particle. The ability of BTV CLPs to bind external nucleic acid may have a role at an early stage in morphogenesis, during the incorporation of ssRNA into a replicative intermediate, or during assembly of VP3 onto a preformed RNA-protein complex.

Since brome mosaic virus (BMV) virions contain only BMV RNAs it is evident that specificity of interaction exists, otherwise, nonviral RNAs and nonviral proteins would be present. In studies, focusing on BMV RNA-1, it was shown that in addition to specific binding, nonspecific binding occurred. Additionally, coat protein and RNA-1 appear to have a strong affinity for each other, since complexes between them are resistant to dissociation by a high salt concentration. Interestingly, a marked decrease in specific binding was only evident upon extensive 3' sequence deletions. Within the region deleted, two domains were identified that appeared to contribute to binding specificity. The existence of multiple regions exhibiting binding suggests that the

secondary or quaternary structure of the viral RNA contributes to specificity. In case of Sindbis virus, a domain in the region encoding nonstructural protein NSP1 was responsible for specific binding of coat protein to both genomic and defective interfering RNAs. Interestingly, this domain is analogous in position to one of the domains of BMV RNA-1. Nonspecific protein-RNA interactions have been widely found for viral RNAs. It was suggested that a lack of specificity may aid in virion assembly by compaction of the RNA through the neutralisation of the negative charge on the phosphate backbone. In addition to the specific interactions data reveal that BMV coat protein interacts with various RNAs in a nonspecific manner. The cooperative pattern of binding exhibited by BMV coat protein probably accelerates encapsidation through protein-protein interactions after a few molecules of the coat protein bind to a specific region of the RNA. This observation explains the rapid *in vitro* assembly of BMV empty protein shells. A coat protein deletion mutant lacking the first 25 amino acids was unable to package BMV RNA *in vivo* (Duggal & Hall, 1993).

In 1983 Richardson & Furuichi reported that reovirus S3 and S2 mRNAs can potentially hybridise to each other. It is intriguing to speculate that such intermolecular interactions may result in the formation of large RNA networks, each containing a complete set of reovirus genes. The terminal sequences of all 10 reovirus RNA segments indicated that complex interactions between termini can occur.

## **1.9 AIMS**

From the literature it can be seen that the nonstructural protein NS2 of AHSV is phosphorylated and can bind to ssRNA in a nonspecific way. Virus assembly occurs in VIBs and NS2 is the sole component in VIB formation. Because of the fact that NS2 both binds ssRNA and forms VIBs, it is thought that it could be involved in the condensation, selection and replication of viral ssRNA genome segments during encapsidation. If NS2 is to be involved in the selection of the ten different virus specific mRNAs from all the other mRNAs in the cytoplasm and for ensuring that one of each of the virus specific mRNAs is packed in to the virus core, it is assumed that some specific mRNA recognition is involved. The nature of the specific interaction or mRNA recognition is unknown.

In order to obtain more information on the binding of AHSV NS2 to ssRNA this study focussed on the following short term aims:

- a) To determine if the difference between the ability of AHSV NS2 and BTV NS2 to bind nonspecifically to poly(U)Agarose can be ascribed to differences in the  $\alpha$ -helix rich C-terminal of NS2.
- b) To prepare individual AHSV mRNAs with authentic ends by constructing a series of recombinant transcription vectors. These mRNAs might enable us to carry out further studies to determine whether NS2 has any preference for AHSV specific mRNAs over nonspecific mRNAs.

These answers will broaden our understanding of the functions of NS2 and how the protein performs its functions in the virus. In the last chapter some concluding remarks will be presented concerning the major findings as well as some suggestions on future investigations.

## CHAPTER 2

# CHARACTERISATION OF THE BINDING ABILITY OF A CHIMERA OF AHSV AND BTV NS2

### 2.1 INTRODUCTION

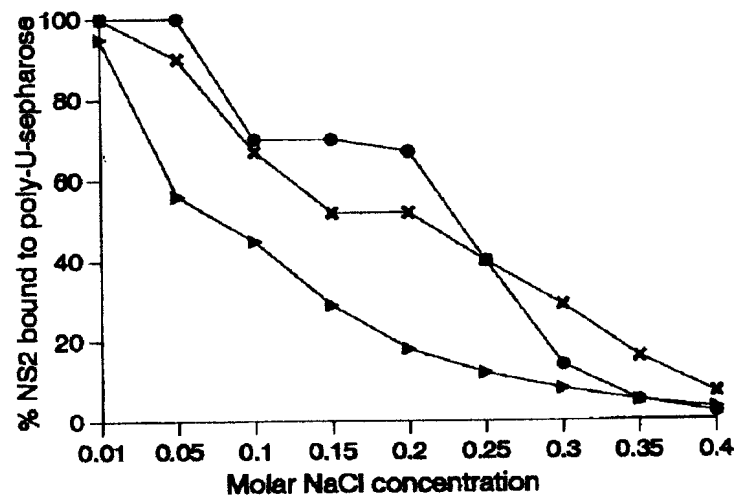
Experimentally the  $\alpha$ -helix has been shown to be the most common fold in proteins, and the conserved helix-turn-helix structural motif of DNA-binding proteins is based on  $\alpha$ -helices interlocking at a large crossing angle over a narrow region (Cohen & Parry, 1986).

Examination of the hydrophilicity profiles and secondary structures of NS2 of EHDV, AHSV, and BTV showed a marked degree of similarity among the proteins. In all three cases the C-terminal region of each protein (in particular amino acids 130-135) was very hydrophilic and was characterised by a high degree of potential  $\alpha$ -helix structure. This region includes amino acids 167-286, which showed the most variation in amino acid sequence among NS2 of the three orbiviruses (average similarity less than 20%) (Van Staden *et al.*, 1991).

RNA binding studies with baculovirus-expressed NS2 of BTV-10 indicated that ssRNA binding is nonspecific (Thomas *et al.*, 1990). Such nonspecific binding could be associated with the  $\alpha$ -helix-rich C-terminal regions of NS2 of BTV, EHDV, and AHSV. Both arginine and lysine residues are spaced throughout this region (Van Staden *et al.*, 1991). If RNA-binding is associated with the  $\alpha$ -helix-rich C-terminal of NS2, it is noteworthy that, based on secondary structure predictions BTV NS2 has the highest  $\alpha$ -helix content (69%), followed by EHDV NS2 (59%), and AHSV NS2 (47%). These results suggest a correlation between the  $\alpha$ -helix content of the NS2 protein and the nonspecific ssRNA-binding ability of the protein (Uitenweerde *et al.*, 1995).

Significant differences were found in the ability of BTV, EHDV, and AHSV NS2 to bind ssRNA, irrespective of the origin of the template - viral or synthetic. It was found that sucrose-gradient-purified 7S NS2 complexes of BTV, AHSV, and EHDV are able to bind to poly(U)-Sepharose in the presence of low salt. Only at the very lowest NaCl concentration (0.01 M) was the NS2 of all

three orbiviruses quantitatively bound to the poly(U)-Sephadex, except for a small percentage ( $\pm 5\%$ ) of AHSV NS2. As the salt concentrations increased a distinct difference in poly(U)-Sephadex binding was observed between the three different NS2 proteins. BTV NS2 displayed the strongest affinity for poly(U)-Sephadex at all the different salt concentrations and AHSV NS2 the weakest affinity. The result obtained with EHDV NS2 was intermediate between those of BTV and AHSV. These results are summarised in Fig. 2.1 (Uitenweerde *et al.*, 1995).



**Fig. 2.1** Comparison of the ssRNA-binding by NS2 of BTV, AHSV, and EHDV in the presence of NaCl. A graphic representation of the percentage of  $^{35}\text{S}$ -labelled NS2 bound to poly(U)-Sephadex at different NaCl concentrations. AcBTV-10.8 NS2 ( $\bullet - \bullet$ ), AcEHDV-2.8 NS2 ( $x - x$ ), AcAHSV-S.8 NS2 ( $\blacktriangleright - \blacktriangleright$ ) (Uitenweerde *et al.*, 1995).

The aim of this part of the study was thus to determine whether the difference between the ability of AHSV NS2 and BTV NS2 to bind nonspecifically to poly(U)Agarose can be ascribed to the differences in the  $\alpha$ -helix rich C-terminals of the two proteins as there seems to be a correlation between the proteins'  $\alpha$ -helix content and their ability to bind ssRNA.

## 2.2 MATERIALS AND METHODS

### 2.2.1 Plasmids, cells and viruses

The chimeric NS2 gene, cloned into the BamH1 site of pBS (pBSChimera), was provided by M. Stoltz (Department of Genetics, University of Pretoria). As the construction of this chimere has not been described elsewhere, details will be included as part of the introduction to the results.

The recombinant baculoviruses AcBTV-10.8, expressing the NS2 protein of BTV serotype 10, and AcAHSV-9.8, expressing the NS2 protein of AHSV serotype 9, were obtained from Dr. V. van Staden (Department of Genetics, University of Pretoria). Monospecific polyclonal antiserum raised in rabbits against both AHSV-9 NS2 (rabbit  $\alpha$ AHSV NS2) and BTV-10 NS2 (rabbit  $\alpha$ BTV NS2) was available as primary antibodies, also obtained from Dr. V. van Staden. The cell line in this study was *Spodoptera frugiperda* (Sf) cells, Sf9. The Sf9 cells were grown as monolayers or suspension cultures in Grace's medium (Highveld Biological) with 10% (v/v) foetal calf serum (Highveld Biological). Wild-type baculovirus (AcNPV) and recombinant baculoviruses were grown and assayed in confluent monolayers of Sf9 cells. Cells and virus were cultured according to the procedures described by Summers and Smith, (1987) and O'Reilly *et al.*, (1992). The AcNPV and Sf9 cells were obtained from the NERC Institute of Virology and Environmental Microbiology, Oxford, UK. Plasmids were propagated in XL1-Blue *E. coli* cells grown in LB-medium (1% bacto-tryptone, 0.5% bacto-yeast extract and 1% NaCl pH 7.4).

### 2.2.2 Preparation of competent *E. coli* cells

XL1-Blue *E. coli* cells were made competent for transformation by adding 1ml of an overnight culture of XL1-Blue cells to 100 ml of pre-warmed sterile LB-medium. The overnight culture was then grown at 37°C with shaking until log phase of growth was achieved at an OD<sub>550</sub> of 0.5. 20 ml of the cultured cells was then collected by centrifugation at 1 500 g for 3 min, gently resuspended in 10 ml of freshly made ice-cold 50 mM CaCl<sub>2</sub> and left on ice for 1 h. Cells were centrifuged again and resuspended in 1 ml of 50 mM CaCl<sub>2</sub> and left on ice for 1 h prior to transformation (see section 2.2.3).

### 2.2.3 Transformation of competent cells

Plasmid DNA was added to 200  $\mu$ l of competent *E. coli* cells in a sterile test tube and left on ice for 30 min. The transformation mixture was subject to a heat shock of 42°C for 90 s allowing the DNA to enter the cells, after which the cells were cooled on ice for 2 min. Pre-warmed LB-medium (0.8 ml) was added before the cells were incubated for 1 h at 37°C with shaking to allow expression of the plasmid encoded antibiotic resistance genes. Aliquots of 100-150  $\mu$ l of the cell culture were plated onto 1.5% (w/v) LB-agar plates containing appropriate antibiotics and incubated inverted at 37°C overnight. Antibiotics commonly included in the plates were ampicillin (100  $\mu$ g/ml agar) and tetracyclin (12.5  $\mu$ g/ml). 50  $\mu$ l of 2% (w/v) X-Gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside, Boehringer Mannheim) and 10  $\mu$ l of 100 mM IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside, Boehringer Mannheim) were added per plate in the case of plasmid vectors with blue-white selection to obtain colour selection. The white colonies would then be recombinants because of the inactivation of the *lac Z* gene, due to insertion of foreign DNA into an appropriate cloning site in the multiple cloning site (MCS) of the vector.

### 2.2.4 Plasmid purification

Plasmids were purified by the rapid alkaline lysis method which is a modification of the method of Birnboim and Doly (1979) as modified by Sambrook *et al.* (1989). Small scale extractions were done on overnight bacterial cultures where a single colony was picked from an agar plate and used to inoculate 3 ml LB-medium. For large scale extractions 100 ml of LB-medium was infected with 1 ml of overnight bacterial culture. The culture was grown overnight at 37°C with shaking. In both cases the plasmid extractions were done in the same way, large scale extractions only used upscaled volumes which will be indicated in brackets. 3 ml (100 ml) of the bacterial culture was harvested, concentrated by low speed centrifugation (1 500 g for 2 min) and resuspended in 100  $\mu$ l (10 ml) of an isosmotic Solution 1 containing 50 mM glucose, 10 mM Na,EDTA, 25 mM Tris pH 8 and left at room temperature for 5 min, followed by 1 min on ice. The resulting spheroplasts were lysed by the addition of 200  $\mu$ l (20 ml) of 0.2 M NaOH, 1% SDS followed by 5 min on ice. This results in the irreversible denaturation of linear chromosomal DNA by the alkali, the closed circular plasmid DNA strands however are unable to separate because they are topologically intertwined. 150  $\mu$ l (15 ml) 3 M NaAc pH 4.8 was added and left for 30 min on ice which caused the closed



circular plasmid DNA to renature rapidly. The denatured proteins were removed along with the chromosomal DNA, by centrifugation for 10 min at 17 000 g. Plasmid DNA was precipitated from the supernatant fluid by the addition of 2 volumes 96% ethanol for 1 hour at -20°C and centrifugation for 10 min at 17 000 g. The DNA pellet was washed with 70% ethanol, freeze dried and resuspended in ultra high quality double distilled water (UHQ). Low molecular mass RNA was removed by precipitation in the presence of 3 M ammonium acetate. After an hour on ice, the precipitate was removed by centrifugation at 17 000 g for 10 min. The plasmid DNA was again precipitated from the supernatant fluid by the addition of 2 volumes ethanol as before, washed in 70% ethanol, dried and resuspended in UHQ.

### 2.2.5 Agarose gel electrophoresis

DNA samples were analysed on 35 ml horizontal slab gels of 1% (w/v) agarose (Seaplaque<sup>R</sup>) in 1 × TAE buffer containing 1.5 µl of 10 mg/ml ethidium bromide. Electrophoresis was performed at 120 V in 1 × TAE buffer in a Biorad Mini Sub<sup>TM</sup> agarose gel electrophoresis unit. The ethidium bromide stained DNA was visualised on a UV transilluminator. DNA molecular weight markers SMII (lambda DNA digested with *Hind III*) and phiX (phiX174 DNA digested with *Hae III*) were used, both from Boehringer Mannheim.

### 2.2.6 Restriction endonuclease digestion

In each case the restriction enzyme digests were performed at 37°C for 1 or more hours with the manufacturer's recommended buffer. At least 1 U enzyme (Boehringer Mannheim) per µg of template was used. The products were analysed by agarose gel electrophoresis (2.2.5).

### 2.2.7 Purification of DNA fragments from agarose gels

The GeneClean<sup>TM</sup> (Bio101 Inc) method was used to purify DNA fragments from agarose gels according to the manufacturer's instructions. DNA fragments were excised from the gel and 2.5 volumes of the provided 6 M NaI was added to lower the melting temperature of the agarose. The agarose was then melted at 55°C. 5 µl of a silica matrix solution (Glassmilk<sup>R</sup>) was added and the DNA was allowed to bind to the Glassmilk<sup>R</sup> by incubating the mixture on ice for 15 min. The silica-

DNA complex was then pelleted by brief centrifugation and the pellet was washed 3× in NEW Wash (NaCl, Tris, EDTA, water and ethanol). The purified DNA was eluted from the silica in a final volume of 10 µl diethyl pyrocarbonate-ultra high quality deionised distilled water (DEPC-UHQ) by incubation at 55°C for 3 min.

#### 2.2.8 Dephosphorylation of linearised vectors

In order to prevent the linearised vectors from recirculating, the digested vector DNA was dephosphorylated. Following linearisation of the vector DNA by restriction enzyme digestion (see section 2.2.6), 1 U of calf intestinal phosphatase (1 U/µl, Boehringer Mannheim) was added together with 5 µl of 10x buffer (50 mM Tris.HCl (pH 8.5), 0.1 mM EDTA) to the restriction enzyme mixture. The reaction volume was adjusted to a final reaction volume of 50 µl with UHQ. The mixture was incubated at 37°C for 10 min followed by the denaturation of the phosphatase at 55°C for 15 min. Electrophoresis followed and the DNA was recovered by GeneClean™ purification.

#### 2.2.9 Ligation of DNA fragments

As the T4 DNA ligase is sensitive to contaminants, the DNA fragments were purified by GeneClean™ prior to ligation. Ligation reactions were made up of purified insert, purified linear vector, T4 ligase buffer (66 mM Tris-HCl pH 7.5, 5mM MgCl<sub>2</sub>, 1 mM DTT, 1 mM ATP) and 1 U T4 DNA ligase (Boehringer Mannheim). The ratio of vector to insert was in excess of 1:2 and a final reaction volume of 15 µl was made up with UHQ. The reaction mixture was incubated at 16°C for 16 h.

#### 2.2.10 Expression of proteins in insect cells with the BAC-TO-BAC™ baculovirus expression system

##### 2.2.10.1 Insertion into pFASTBAC1

The donor vector, pFASTBAC1, contains the strong polyhedrin promoter followed by an extensive MCS. The original ATG start codon of the polyhedrin gene has been mutated to ATT. Because of this mutation the foreign DNA fragment must contain its own ATG initiation codon followed by an

open reading frame (ORF) in order to express the foreign protein successfully. It is therefore essential that the foreign gene must be cloned in the correct orientation with respect to the polyhedrin promoter. The foreign gene is in the correct orientation if the 5' of the gene is inserted into the first selected site of the MCS (Van Oers *et al.*, 1999). The foreign DNA as well as the donor vector, which was first purified from cells as described in section 2.2.4, were digested with the restriction endonuclease of choice as previously described in section 2.2.6. After the vector was dephosphorylated as discussed under section 2.2.8, both the vector and the DNA fragment were recovered from gels using the GeneClean™ method (section 2.2.7). Ligation occurred under appropriate conditions (section 2.2.9) and *E. coli* XL1-Blue cells were transformed with the ligation mixture (section 2.2.3). After incubation overnight at 37°C on LB-agar plates containing ampicillin and tetracycline several independent clones were selected for analysis, grown up in liquid culture overnight and analysed by small scale plasmid purification and electrophoresis (see sections 2.2.4 and 2.2.5). The orientation of the fragment in the plasmid was determined by restriction enzyme digestion and clones with the gene in the correct orientation were selected for transposition into the bacmid.

#### 2.2.10.2 Transposition

The pFASTBAC1 recombinant plasmid carries a Tn7 transposition element containing a gentamycin resistance gene, polyhedrin promoter, foreign gene sequence and a SV40 poly (A) sequence. Autonomous replication in *E. coli* by the bacmid genome is possible because it contains a mini-F replicon. The bacmid genome also contains an *attTn7* transposition site, a kanamycin resistance gene and a *lac Z $\alpha$*  complementation region. The Tn7 element on the donor plasmid is able to transpose into the *attTn7* site on the bacmid when the Tn7 transposition functions are provided *in trans* by the helper plasmid that is tetracycline resistant. DH10BAC™ cells containing the bacmid genome and helper plasmid were grown up overnight and made competent using the DMSO method (Chung & Miller, 1988). 100  $\mu$ l of the competent DH10BAC™ cells were dispensed into a test tube and approximately 0.1 - 0.5  $\mu$ g of the recombinant pFASTBAC1 plasmid with the foreign gene in the correct orientation was added. The mixture was incubated on ice for 30 min and then heat shocked at 42°C for 45 s. Lastly 900  $\mu$ l of SOC medium (LB-agar to which is added 10% (w/v) PEG, 5% (w/v) DMSO, 10 mM MgCl<sub>2</sub>, 10 mM MgSO<sub>4</sub>) was added and the mixture was incubated with shaking for 4 h at 37°C. During this incubation period transposition occurred and

the plasmid-encoded antibiotic resistance genes were expressed. LB-plates containing 50 µg/ml kanamycin sulphate, 7 µg/ml gentamycin, X-Gal and IPTG as for section 2.2.3 were prepared and 100 µl of the culture medium was plated out per plate. After 24 h of inverted incubation blue and white colonies could be distinguished from each other. Blue colonies represent colonies in which transposition did not occur as the bacmid genome carries the *lac Zα* gene. If transposition did not occur and the bacmid genome is transformed into *lac* DH10BAC™ cells, a functional β-Galactosidase protein is formed and the cells produce a blue colour on plates containing X-Gal and IPTG. When transposition of the Tn7 element into the bacmid genome by the pFASTBAC1 plasmid does occur the *lac Zα* gene is disrupted, the β-Galactosidase protein is no longer formed and the recombinant colonies appear white on plates containing X-Gal and IPTG. Several recombinant white colonies were selected and in order to determine if they were indeed true white colonies they were restreaked onto fresh LB-agar plates containing antibiotics and histochemicals as previously described. A single true white colony was then picked up, using a sterile toothpick, and was inoculated into liquid LB-medium supplemented with kanamycin and gentamycin. The inoculum was incubated at 37°C for 16 h prior to isolation of composite bacmid DNA.

#### 2.2.10.3 Isolation of composite bacmids

In order to isolate the composite bacmid DNA, 1 ml of the liquid culture, grown up overnight, was spun down at maximum speed in a microcentrifuge for 1 min. After the supernatant was removed, the pellet was resuspended in 300 µl Solution 1 (see section 2.2.4). Solution 2 (containing 0.2 N NaOH, 1% SDS) was added (300 µl) and the contents of the tube was gently mixed by inverting the tube. The reaction mixture was then incubated at room temperature for 5 min. 300 µl of a 3 M KAcetate pH 5.5 solution was slowly added while the mixture was again gently mixed during addition. The sample was then placed on ice for 10 min and centrifuged at 17 000 g for 10 min in a microcentrifuge. The supernatant was then gently transferred to 800 µl isopropanol, avoiding the white precipitate, and incubated for 10 min on ice. After another 15 min of centrifugation at maximum speed at room temperature the supernatant was removed and the DNA was washed with 70% ethanol. The pellet was air-dried and resuspended in 40 µl UHQ prior to transfection.

#### 2.2.10.4 Transfection of Sf9 cells with bacmid DNA

Following isolation of the recombinant bacmid DNA,  $9 \times 10^5$  Sf9 cells per well were seeded in 35 mm six-well tissue culture plates (Nunclon™). The cells were allowed to attach to the tissue culture plates for 1 h at 27°C. The two transfection solutions, Solution A and Solution B were prepared while the cells attached. Solution A consisted of 6 µl of recombinant bacmid DNA (obtained in 2.2.10.3) that was diluted into 100 µl Grace's medium. Grace's medium without serum or antibiotics was used as both could inhibit uptake of the exogenous DNA. Solution B consisted of 6 µl of an optimised liposomal mixture, CELLFECTIN™ Reagent, in 100 µl Grace's medium without antibiotics. The two solutions were combined, gently mixed and incubated at room temperature for 45 min. After the cells were attached they were washed with 2 ml Grace's medium without serum or antibiotics. For each transfection 800 µl of Grace's medium was added to each tube containing the lipid-DNA complexes. The wash medium was aspirated and the washed cells were overlaid with the diluted lipid-DNA complexes. In order to allow uptake of the recombinant bacmid DNA the cells were incubated at 27°C for 5 h after which the transfection mixtures were removed and 2 ml Grace's medium containing serum and antibiotics was added. After another 96 h of incubation at 27°C the recombinant virus was harvested for further infections and protein expression.

#### 2.2.11 Preparation of virus stocks and protein expression

Sf9 cells were seeded in 75 mm tissue culture flasks (Nunclon™) at a density of  $9 \times 10^7$  and were allowed to attach for 1 h at 27°C. After attachment the Grace's medium was removed and the cells were infected with viral inoculum at the desired multiplicity of infection in a total volume of 6 ml. The cells were then incubated for another hour at 27°C to allow the adsorption of the virus. 9 ml of medium was added to make up the volume to a total of 15 ml where after the cells were further incubated at 27°C for another 4 days. For virus stocks the supernatant was poured off and the cellular debris removed by low speed centrifugation, 15 min at 1 500 g. The supernatant was then filtrated to remove any other contaminants and stored at 4°C for further use. For protein expression the cells were harvested, washed twice in 1 × PBS (137 mM NaCl; 2,7 mM KCl; 4,3 mM Na<sub>2</sub>HP0<sub>4</sub>.2H<sub>2</sub>O; 1,4 mM KH<sub>2</sub>PO<sub>4</sub>) and stored at -20°C until use.

### 2.2.12 SDS-PAGE analysis

Protein samples were analysed by SDS-PAGE according to the discontinuous buffer system (Laemmli, 1970). The gel consists of two parts, a 5% acrylamide: 0.13% bisacrylamide (w/v) stacking gel and a 12% acrylamide:0.32% bisacrylamide (w/v) separating gel. The separating gel was set on the bottom and consisted of acrylamide:bisacrylamide, 0.375 M Tris.HCl pH 8.8, 0.1% SDS and was polymerised by addition of 0.06% (v/v) ammonium persulphate and 0.06% (v/v) TEMED. The separating gel was then overlaid with water saturated butanol and allowed to polymerise. After polymerisation of the separating gel the butanol was removed and the stacking gel was set on top. The stacking gel consisted of acrylamide:bisacrylamide, 0.125 M Tris.HCl pH 6.8, 0.1% SDS and was polymerised by addition of 0.16% (v/v) ammonium persulphate and 0.6% (v/v) TEMED. The stacking gel was allowed to polymerise after which electrophoresis was performed in 7x10 cm Hoefer Mighty Small™ electrophoresis units. The running buffer was TGS and consisted of 0.025 M Tris pH 8.3, 0.192 M glycine, 0.1% SDS. Prior to loading, the samples were mixed with an equal volume of 2 x PSB (Protein solvent buffer - 0.125 M Tris pH 6.8, 4% SDS, 20% glycerol, 10% β-mercapto-ethanol) and heated at 95°C for 3 min. Electrophoresis was performed at 120 V for 2 hours. The gels were then stained in 0.125% Coomassie Blue, 50% methanol, 10% acetic acid for 20 min and destained in 5% acetic acid and 5% methanol overnight. Rainbow™ protein molecular weight size markers (MW 14.300-200.000) (Amersham) were used.

### 2.2.13 [<sup>35</sup>S]-methionine labelling of proteins *in vivo*

Sf9 cells were seeded in 75 mm tissue culture flasks (Nunc™) and infected at a high multiplicity of infection (in order to ensure synchronised infections) with the various baculovirus recombinants and incubated at 27°C for 29 h. The Grace's medium was poured off and replaced with 10 ml of methionine-free Eagle's medium (Highveld Biological). In order to use all the intracellular methionine the cells were then incubated for 1 h. The Eagle's medium was then replaced with 5 ml of fresh Eagle's medium containing 30 μCi of [<sup>35</sup>S]-methionine (Amersham) and incubated for another 3 h to ensure the incorporation of the radio active methionine into the proteins. After incubation the cells were harvested, washed in 1 x PBS and stored at -20°C until use. To see how well the cells were labelled, samples were denatured and analysed by SDS-PAGE (section 2.2.12). The gels were then fixed in 7% acetic acid and dried on a slab gel-dryer (Hoefer Scientific

Instruments) for 1 h at 80°C under vacuum. Lastly the gels were exposed to Cronex MRF31 X-ray film at room temperature for at least 3 days before developing.

#### 2.2.14 Western immunoblot analysis

In order to perform the immunoblot analysis the samples were first analysed by SDS-PAGE (see 2.2.12). The gel and an equally sized piece of nitrocellulose (Hybond C+, Amersham), together with 12 sheets of filter paper, also the same size as the gel, were soaked for 20 min in transfer buffer (25 mM Tris, 150 mM glycine pH 8.3, 20% methanol) prior to blotting. The proteins were transferred to the nitrocellulose membrane by electroblotting in a BioRad Trans-Blot<sup>R</sup> Semi-Dry Electrophoretic Transfer Cell at 3 mA/cm<sup>2</sup> gel. After the proteins were transferred to the nitrocellulose, non-specific absorption of the immunological reagents was prevented by incubating the membrane in a solution of 1% (w/v) fat free milk powder in 1×PBS at room temperature for 30 min. After incubation the blocking solution was poured off and the monospecific polyclonal antiserum raised in rabbits against both AHSV-9 NS2 (rabbit αAHSV NS2) and BTV-10 NS2 (rabbit αBTV NS2) were diluted in blocking buffer (1/1000) and added as primary antibodies. The membranes were then incubated overnight at room temperature with gentle shaking. The next morning the membrane was washed with 3 changes of wash buffer (0.05% Tween<sup>R</sup>- 20 in 1×PBS) for 5 min each. Protein A conjugated with horseradish peroxidase (Cappel), was diluted in blocking buffer (1/1000), and added to the membrane as secondary antibody, and incubated at room temperature for 1 h with gentle shaking. Again the membranes were washed 3 times in washing buffer and once in 1×PBS. After they were washed the membranes were transferred to the enzyme substrate (60 µg 4-chloro-1-naphtol dissolved in 20ml ice-cold methanol mixed just prior to use with 100ml 1×PBS containing 60µl H<sub>2</sub>O<sub>2</sub>). The colour reaction was allowed to develop for a few minutes before the blot was rinsed in water, to prevent unacceptable background, and air dried.

#### 2.2.15 Sedimentation analyses

Cytoplasmic fractions were prepared from  $1 \times 10^7$  Sf9 cells infected with the respective NS2 recombinant baculovirus at a multiplicity of infection of 10 pfu/cell. The cells were twice washed in 1 ml of 1×PBS. After the cells were washed they were resuspended in 500 µl 0.15 M STE-TX

(0.15 M NaCl, 10 mM Tris, pH7.4, 1 mM EDTA, 0.5% Triton X-100) and incubated for 30 min on ice. The cells were then sonicated and centrifuged for 5 min at 1 000 g to spin down the nuclei. The supernatants were loaded on 10-40% sucrose gradients in 0.15 M STE-TX and centrifuged for 16 h at 200 000 g (SW50 rotor). Forty drops per fraction was collected from the bottom of the gradients and the fractions containing the 7S NS2 multimer were identified by SDS-PAGE (section 2.2.12) followed by Western-blot analysis (section 2.2.14).

#### 2.2.16 Poly(U)-Agarose binding assay

For the Poly(U)-Agarose binding assay, cytoplasmic fractions were prepared from  $1 \times 10^7$  Sf9 cells infected with the respective NS2 recombinant baculovirus at a high multiplicity of infection, of at least 5 pfu/cell, in order to ensure synchronized infections. The proteins were then labelled with [<sup>35</sup>S]- methionine at 30-33 h postinfection (section 2.2.13). The cells were then harvested and washed twice in 1×PBS. Hereafter the cells were resuspended in 500 µl 0.5 M STE-TX (0.5 M NaCl, 10 mM Tris, pH7.4, 1 mM EDTA, 0.5% Triton X-100) and incubated for 30 min on ice. After incubation, the cells were sonicated and the nuclei were spun down by centrifugation for 5 min at 1000 g. The supernatants were loaded on 10-40% sucrose gradients in 0.4 M STE-TX (0.4 M NaCl, 10 mM Tris, pH7.4, 1 mM EDTA, 0.5% Triton X-100) and centrifuged for 16 h at 200 000 g (Beckman SW50 rotor) to obtain unbound NS2. Forty drops per fraction were collected from the bottom of the gradients and the fractions containing the 7S NS2 multimer were identified by SDS-PAGE (section 2.2.12) and autoradiography (section 2.2.13). The fractions were also double checked for the presence of NS2 by Western-blot analysis (section 2.2.14). The appropriate fractions were pooled and used to assay RNA-binding. The assays were performed in the presence of excess amounts of poly(U)-Agarose (Sigma). 30 µl of the pooled sucrose gradient fractions was added to 20 mg poly(U)-Agarose in 500 µl STE-TX, pH 7.5, at nine different NaCl concentrations of between 0.01 M and 0.4 M as indicated in the results. Binding between the proteins and the poly(U)-Agarose was allowed to occur for 1 h at 4°C with gentle agitation. The poly(U)-Sepharose was collected by centrifugation for 1 min at 1 000 g and washed twice in the appropriate binding buffer. Both the bound and the unbound proteins were quantitated by scintillation counting.



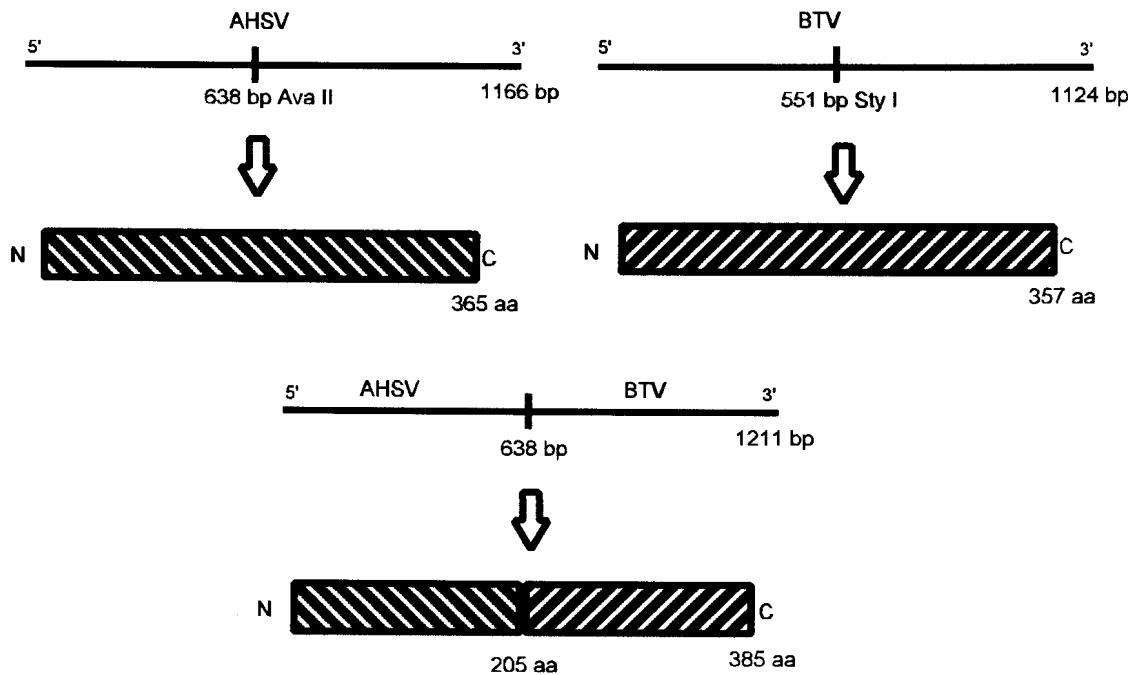
## 2.3 RESULTS

In order to provide an answer to the question whether NS2's ssRNA binding ability is due only to the  $\alpha$ -helix content or if other factors also play a role, we were kindly provided with a chimera that consists of the 5' end of the AHSV NS2 gene and the 3'  $\alpha$ -helix rich end of the BTV NS2 gene, constructed by M.A. Stoltz in the Department of Genetics, University of Pretoria. The binding ability of the chimeric NS2 protein can now be compared to that of both AHSV and BTV NS2. If the chimeric NS2 protein's binding ability is the same as that of the BTV NS2 it could be proposed that the  $\alpha$ -helix content is the only role player in binding affinity. If it is not the case other factors must also play a role in determining binding affinity.

The chimeric NS2 protein was constructed using AHSV serotype 9 S8 and BTV serotype 10 S8. Both genes were first subjected to PCR using primers that were constructed in such a way that they contained *Bam* *HI* restriction sites at both ends. As Fig. 2.2 demonstrates, the 1166 bp AHSV NS2 gene was cut with the restriction enzyme *Ava* *II* between base pair numbers 638 and 639. The 1124 bp BTV NS2 gene, on the other hand, was cut between base pairs 551 and 552 with the restriction enzyme *Sty* *I*. As *Ava* *II* and *Sty* *I* do not share the same restriction overhangs, the overhangs were filled using the Klenow enzyme. The fragment from the AHSV NS2 gene, base pairs 1 to 638, consisting of the N-terminal of the gene was blunt-end ligated to the 573 bp BTV NS2 gene fragment consisting of the  $\alpha$ -helix rich C-terminal to form the 1211 bp gene encoding for the chimeric protein. Sequencing of the chimeric gene confirmed correct ligation and the presence of an ORF from the AHSV initiation codon to the BTV termination codon. This chimeric gene was then ligated to the *Bam* *HI* digested fragments of the pBS vector.

This chimeric protein could subsequently be expressed in the novel BAC-TO-BAC™ baculovirus system (Life Technologies), and compared to the already expressed AHSV NS2 and BTV NS2 proteins. In this high-level eukaryotic expression system (pre-empted by Luckow *et al.*, 1993), the protein of interest is incorporated into the baculovirus genome and this recombinant baculovirus then infects its natural host cells i.e. *Spodoptera frugiperda* (Sf9) insect cells. The novel BAC-TO-BAC™ baculovirus protein expression system differs from the conventional baculovirus expression system in that all the site-specific transposition and selection procedures, previously performed in insect cells, are now carried out in easily sustainable *E. coli* bacteria. This method

greatly increases the speed and efficiency with which recombinant baculoviruses are produced.



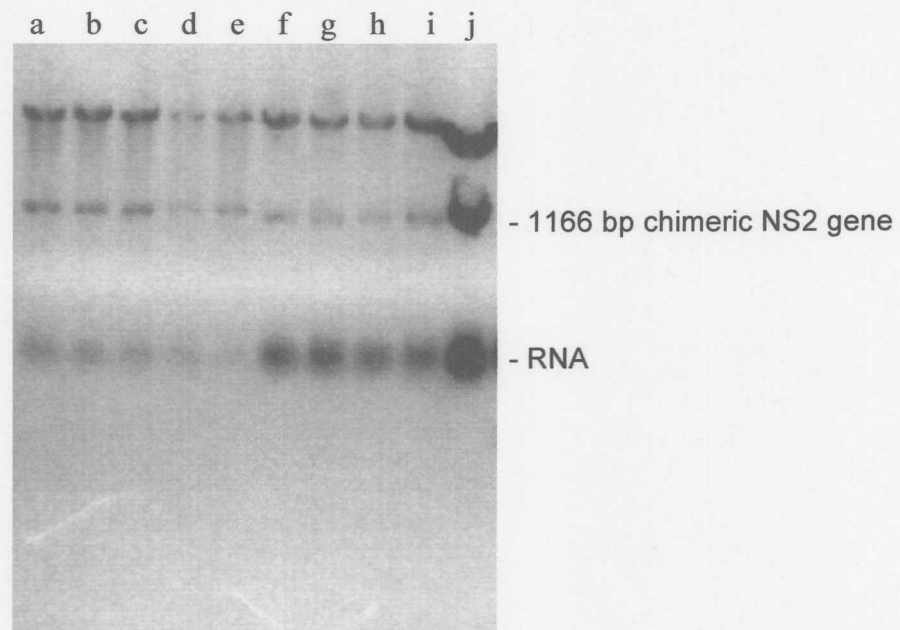
**Fig. 2.2** Construction of a chimeric NS2 gene and protein

### 2.3.1 Baculovirus expression of a chimeric AHSV/BTV NS2 gene

#### 2.3.1.1 Preparation of a pFASTBAC1 recombinant with the AHSV/BTV NS2 chimera

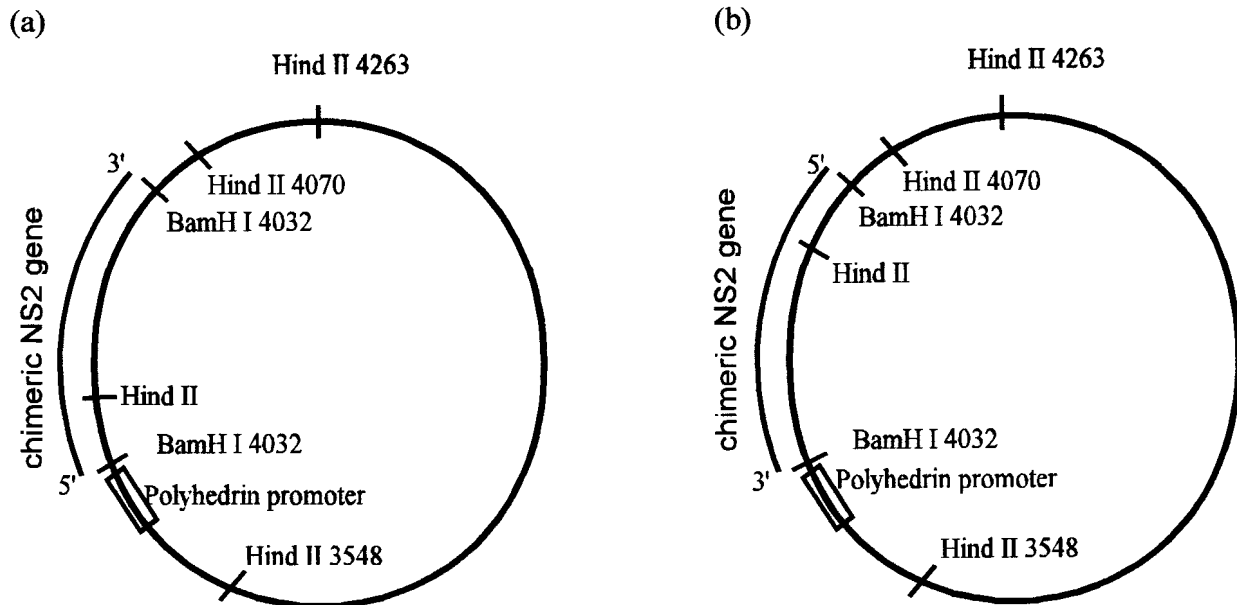
The pBS vector containing the chimeric NS2 gene, designated pBSChimera (M.A. Stoltz), was constructed as summarised in Fig. 2.2. The pBSChimera vector was purified by a large scale plasmid isolation and the chimeric NS2 gene was then excised from the vector through digestion with *Bam* *HI*. Both the NS2 chimera and the *Bam* *HI* linearised and dephosphorylated pFASTBAC1 vector were glassmilk purified after electrophoresis on 1% agarose gels (results not shown). After DNA purification a ligation reaction was set up which consisted of insert and vector in a ratio of 3 insert : 1 vector. Ligation was allowed to occur overnight. *E. coli* XL1-Blue cells were made competent and the ligation mixture was transfected into the cells. The transformed cells were then

plated out onto LB-agar plates and incubated overnight. Since the pFASTBAC1 system does not have any selection system to determine which cells were transformed with plasmids containing the chimeric NS2 genes, numerous colonies had to be screened by plasmid extractions. Plasmids were analysed by agarose gel electrophoresis and the samples that appeared to contain inserts were selected and digested with *Bam* *HI*. *Bam* *HI* digested pBSChimera was used as a control as it would provide us with the 1166 bp chimeric NS2 gene that could be used as a size marker. It can be seen that a fragment of the expected size was excised from all nine recombinants, indicating the successful ligation of the insert (Fig. 2.3).



**Fig. 2.3** Agarose gel electrophoresis analysis of pFASTBAC1-chimeric NS2 recombinant plasmids. Recombinant plasmids (a-i) were digested with *Bam* *HI* and compared to *Bam* *HI* digested pBSChimera (j).

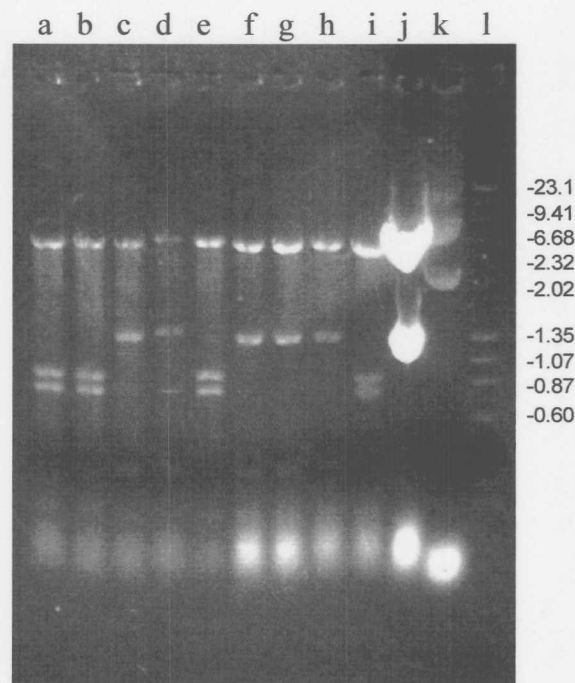
It was subsequently necessary to determine which of the clones had the insert in the correct orientation with respect to the transposition sites. The chimeric NS2 gene had to be in the right orientation for protein expression before transposition could occur in order to obtain a functional recombinant baculovirus.



**Fig. 2.4** Orientation determination in pFASTBAC1-chimeric NS2. (a) Gene in the correct orientation. (b) Gene in the incorrect orientation.

The orientation was determined by restriction mapping. The chimera consists of the 5' end of AHSV NS2 and the 3' end of BTV NS2 and contains a *Hind II* site between base pairs 324 and 325. The chimera also has a *Sma I* site between base pairs 711 and 712 and a *Pvu I* site between base pairs 770 and 771. The vector, pFASTBAC1, on the other hand also has three *Hind II* restriction sites between base pairs 3548 and 3549, 4070 and 4071 and between base pairs 4263 and 4264, but no restriction sites for either *Sma I* or *Pvu I*. The gene inserted into the *Bam HI* site of the vector can be in one of two orientations as illustrated in Fig. 2.4. In the correct orientation, Fig. 2.4(a), the *Hind II* site in the chimeric NS2 gene fragment will be closest to the 3548 base pairs *Hind II* site in the vector, and in the wrong orientation closest to the 4070 base pairs *Hind II* site. Thus if the plasmid with the chimeric NS2 gene fragment is digested with *Hind II*, it will yield four fragments of sizes 4060, 925, 808 and 193 base pairs when in the correct orientation, while it will yield four fragments of sizes 4060, 1371, 362 and 193 when in the incorrect orientation.

Results of the *Hind II* digestion of the nine recombinant plasmids can be seen in Fig. 2.5. The four recombinants that were found to have the gene in the correct orientation are shown in lanes (a), (b), (e) and (i), as these four lanes contained fragments of sizes approximately 4060, 925, 808 and 193 bp. The recombinants from lanes (c), (d), (f), (g) and (h) were in the wrong orientation as sizes of approximately 4060, 1371, 362 and 193 bp were obtained. The pFASTBAC1 recombinant from lane (e) which contained the chimeric NS2 in the correct orientation (subsequently designated fb-chimera) was used for transformation of DH10BAC™ cells and transposition into the bacmid genome.



**Fig. 2.5** Orientation determination of the chimeric NS2 gene in pFASTBAC by restriction enzyme digestion and agarose gel electrophoresis. pFASTBAC-chimeric NS2 recombinants 1-9 (lanes a-i) were all digested with *Hind II*. Controls were pBSChimera digested with *Bam HI* to show the correct size of the chimeric NS2 gene (j), undigested pFASTBAC1 (k) and size markers with sizes as indicated (l).

### 2.3.1.2 Transposition of fb-chimera into the bacmid genome

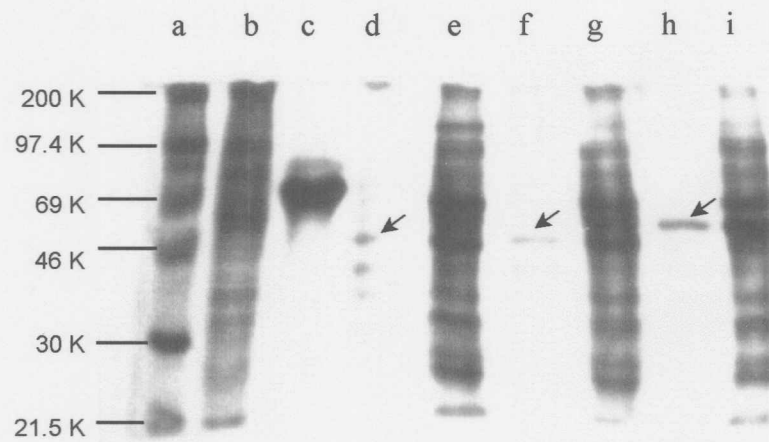
100µl aliquots of fb-chimera were transfected to competent DH10BAC™ cells, that contain the bacmid genome and helper plasmid. Cells were made competent using the DMSO method (section 2.2.10). Following transformation the cells were incubated with shaking at 37°C for 4 h prior to being streaked out on LB-agar plates containing tetracycline, gentamycin, kanamycin, IPTG and X-Gal. The plates were incubated overnight at 37°C and both blue and white colonies were obtained. White colonies were selected at random and were streaked out again on the plates described above to determine if they were indeed true whites. Some of the true white colonies were then selected and grown up in order to isolate the recombinant bacmid genomes from the cells. The isolation procedure is described in section 2.2.10. The isolated bacmid genomes were used directly for the transfection of Sf9 cells as they were too large to be analysed on a 1% agarose gel.

### 2.3.1.3 Isolation and characterisation of baculovirus recombinants containing the NS2 chimere

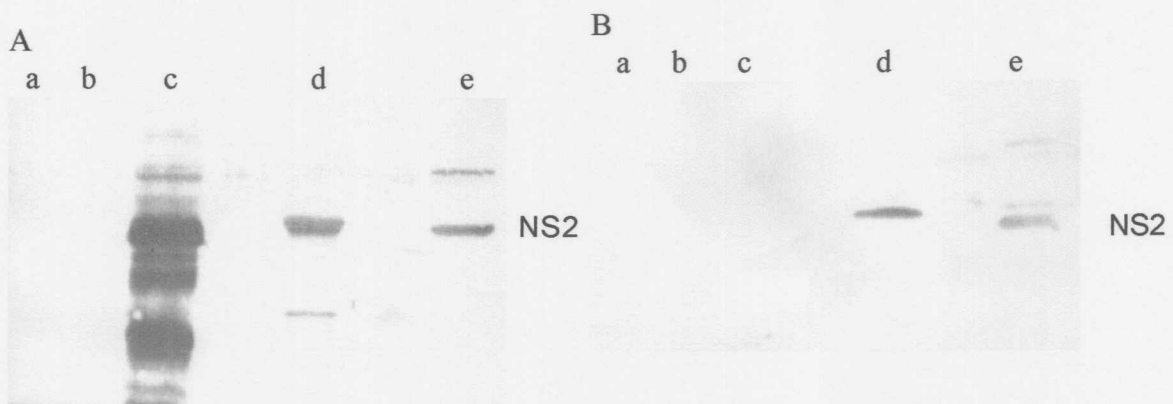
Sf9 cells were seeded in 35 mm six-well tissue culture plates and transfected with the recombinant bacmid DNA samples containing the chimeric NS2 gene as described in section 2.2.10 before the cells were incubated. After 96 h the virus containing supernatant fluid was poured off, filter sterilized and stored at -20°C until used. These virus stocks were later used for re-infection of Sf9 cells for virus amplification or for protein propagation. Recombinant baculoviruses containing the chimeric NS2 gene will be referred to as AcChimera. In order to make sure that the virus amplification was successful and that the recombinant baculoviruses expressed the chimeric NS2 protein, a SDS-PAGE analyses was performed. The SDS-PAGE results are shown in Fig. 2.6. The chimeric NS2 protein was run alongside controls consisting of AHSV NS2 and BTV NS2 respectively. For all three proteins 2 µl of sample was loaded followed by 10 µl of the same sample.

Both AHSV NS2 and BTV NS2 migrated at approximately the same rate through the gel as the 46 kDa marker as can be seen in Fig. 2.6, even though they both have a predicted molecular weight of about 41 kDa. If it seems that the proteins migrated at a lower rate than the marker it is because of the gel that is distorted. The chimeric NS2 protein, with a predicted molecular weight of about

43.8 kDa, migrated at a slightly lower rate than the other two proteins. This is probably due to its slightly bigger size.



**Fig. 2.6** SDS-PAGE analysis of proteins produced in Sf9 insect cells. Lane (a) contains rainbow marker (sizes as indicated), lane (b) mock infected cells and lane (c) wild type baculovirus. The other lanes contain recombinant baculovirus containing AHSV NS2 (d and e), BTV NS2 (f and g) and chimeric NS2 (h and i) respectively. In order to increase the visibility of the expressed NS2 proteins, two different quantities were loaded in case of each of the baculovirus recombinants. The arrows indicate the positions of the NS2 genes



**Fig. 2.7** Western-blot analysis of baculovirus expressed NS2 proteins. These analysis were done in duplicate using A) rabbit  $\alpha$ AHSV NS2 antiserum and B) rabbit  $\alpha$ BTV NS2 antiserum. In both cases lanes (a) contain mock infected cells and lanes (b) wild type baculovirus. The other three lanes contain in each case recombinant baculoviruses expressing AHSV NS2 (c), chimeric NS2 (d) and BTV NS2 (e) respectively.

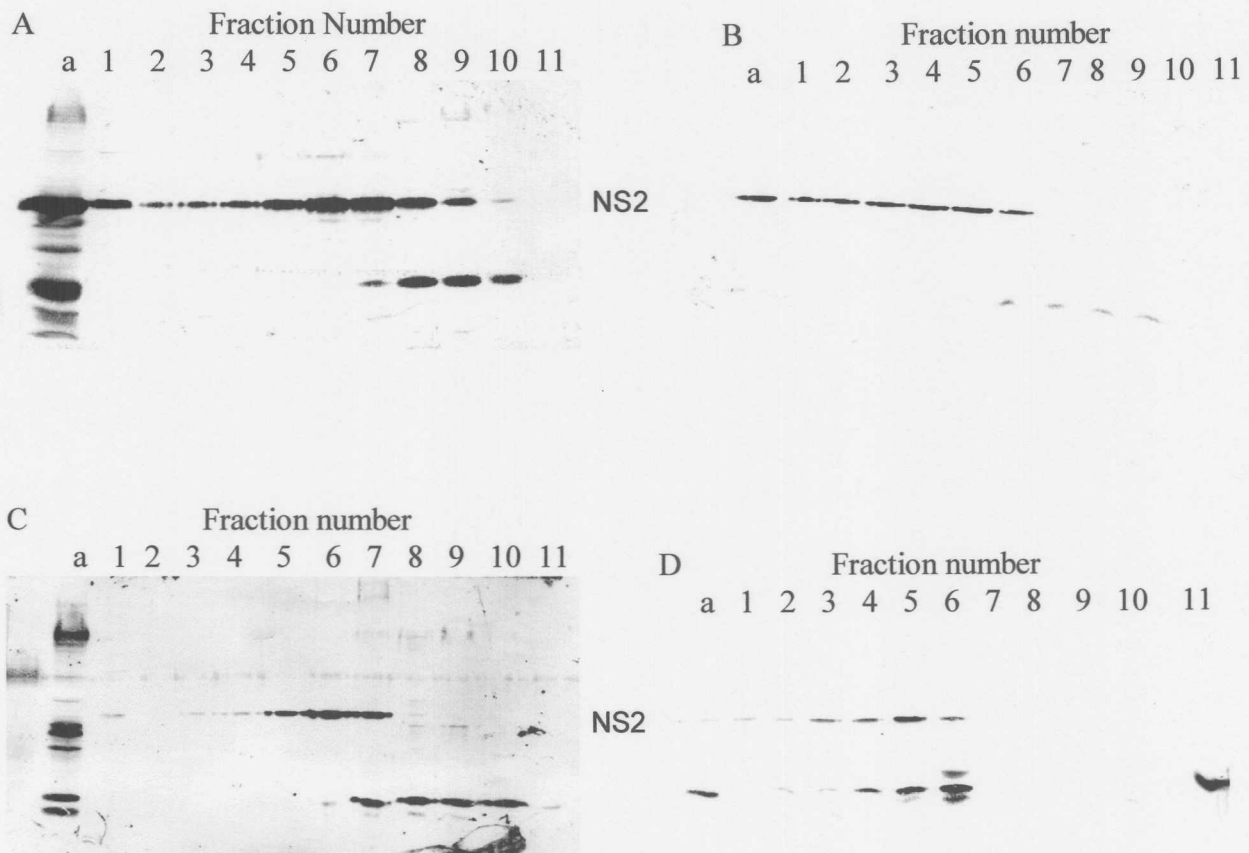
To verify that this protein that migrated slower than AHSV NS2 and BTV NS2 was indeed the chimeric NS2 and not some contaminant, Western blots were performed using monospecific polyclonal antiserum raised in rabbits against both AHSV-9 NS2 (rabbit  $\alpha$ AHSV NS2) and BTV-10 NS2 (rabbit  $\alpha$ BTV NS2) as primary antibodies respectively as seen in Fig. 2.7(A) and (B). Both antisera recognised the chimeric NS2 which migrated slower than AHSV NS2 and BTV NS2. This was expected as the chimera was constructed of both AHSV NS2 and BTV NS2. What is interesting is that while the rabbit  $\alpha$ AHSV NS2 antiserum recognises AHSV NS2, the chimeric NS2 and BTV NS2, the rabbit  $\alpha$ BTV NS2 antiserum only recognises the chimeric NS2 and the BTV NS2 and not the cognate AHSV NS2. We do not have any explanation for all the extra bands in Fig. 2.7A lane (c) as they always occurred whenever the experiment was repeated.

### 2.3.2 Sedimentation analysis of chimeric NS2

The fact that the chimeric NS2 protein migrated slower on a SDS-PAGE gel than the NS2 proteins of AHSV and BTV prompted the question whether the chimeric NS2 was still soluble and if it still had any ssRNA binding ability. In order to answer these questions a sucrose gradient analysis of baculovirus-expressed chimeric NS2 was carried out at low salt concentration. NS2 is known to bind ssRNA at a low salt concentration (Uitenweerde *et al.*, 1995), using low salt conditions would allow the NS2 proteins to bind the cellular mRNA. Cytoplasmic extracts were thus prepared of baculovirus-infected cells in 0.15 M STE-TX and analysed on 10-40% sucrose gradients as described in section 2.2.15. The fractions were collected and analysed by SDS-PAGE and Western blot. Fig. 2.8 represents the Western blot analysis of fractions from gradients of AcAHSV-9.8- (Fig. 2.8A), AcBTV-10.8- (Fig. 2.8B) and AcChimera-infected Sf9 cell lysates (Fig. 2.8C & D).

NS2 expressed by AcBTV-10.8 (Fig. 2.8B), was found as a heterogeneous smear of NS2-containing material from the middle of the gradient to the pellet. NS2 expressed by AcAHSV-9.8 (Fig. 2.8A), however, sedimented with a discreet peak in fractions 6 and 7 of the gradient with a decreasing amount of NS2 in fractions 1 to 5. NS2 of AcChimera sedimented similar to NS2 of AcAHSV-9.8 with also a peak in fractions 6 and 7. The fact that not all of the chimeric NS2 was present in the pellet fraction confirmed that it was still soluble.





**Fig. 2.8** Western blot analyses of the fractions and pellets obtained following sucrose gradient analysis at physiological salt conditions of cytoplasmic extracts of (A) AcAHSV-9.8- using rabbit  $\alpha$ AHSV NS2, (B) AcBTV-10.8- using rabbit  $\alpha$ BTV NS2 antiserum, (C) AcChimera- using rabbit  $\alpha$ AHSV NS2 antiserum, and (D) AcChimera-infected Sf9 cells using rabbit  $\alpha$ BTV NS2 antiserum. The top of the gradients are to the right. The position of NS2 is in each case indicated on the figure. (a) is in each case the unfractionated samples and (1) the pellet.

It is now long been known that NS2 binds ssRNA (Huisman & Basson, 1983) and is associated with ssRNA in the cell. Uitenweerde *et al.*, (1995), have also shown that there is a discreet difference in the ssRNA affinity between NS2 proteins from AcAHSV-9.8- and AcBTV-10.8 infected Sf9 cells. From Fig. 2.8.B it can be seen that BTV derived NS2 binds strongly to cellular ssRNA and thus forms a smear from the pellet. AHSV derived NS2, on the other hand, binds less strongly to cellular ssRNA and forms a peak in fractions 6 and 7. This peak, or concentration of protein,

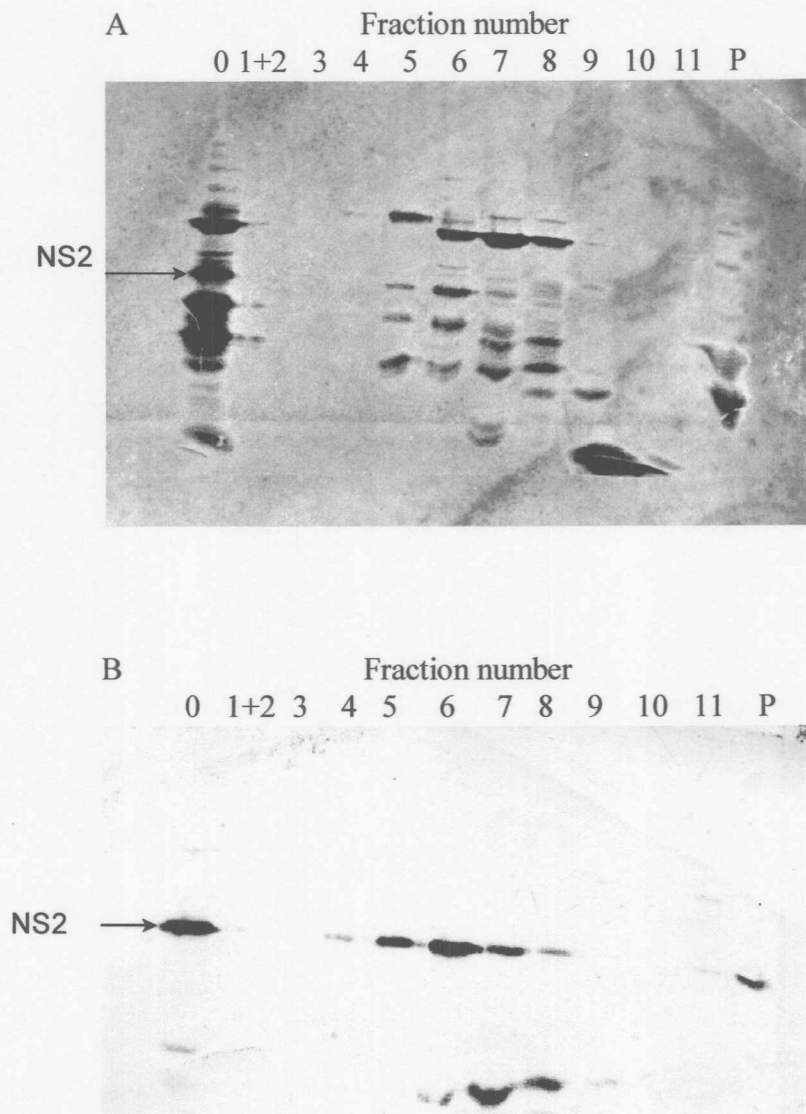
represents unbound NS2 oligomers at a S value of 7S. Here decreasing amounts of bound NS2 form a smear to the pellet. This fact that BTV derived NS2 protein has a much higher affinity for ssRNA than AHSV derived NS2 protein was also shown by Uitenweerde *et al.* (1995). It can also be seen in Fig. 2.8 (C) and (D) that the chimeric NS2 have the same sedimentation profile as the AHSV derived NS2. The fact that there seems to be very little NS2 in lane a for the chimeric NS2 (Fig. 2.8 C & D) is a clear indication that the viral yield was much lower than that of AHSV NS2 (Fig. 2.8 A) where a very clear band is visible in lane a. The amount of chimeric NS2 protein produced was thus a lot less than that for AHSV NS2, therefore all the bands, including the pellet fraction, are a lot lighter than for AHSV NS2. Does this similar sedimentation profile imply similar binding affinity? This will be investigated in the next part of this chapter.

### 2.3.3 Poly(U)-Agarose binding assay

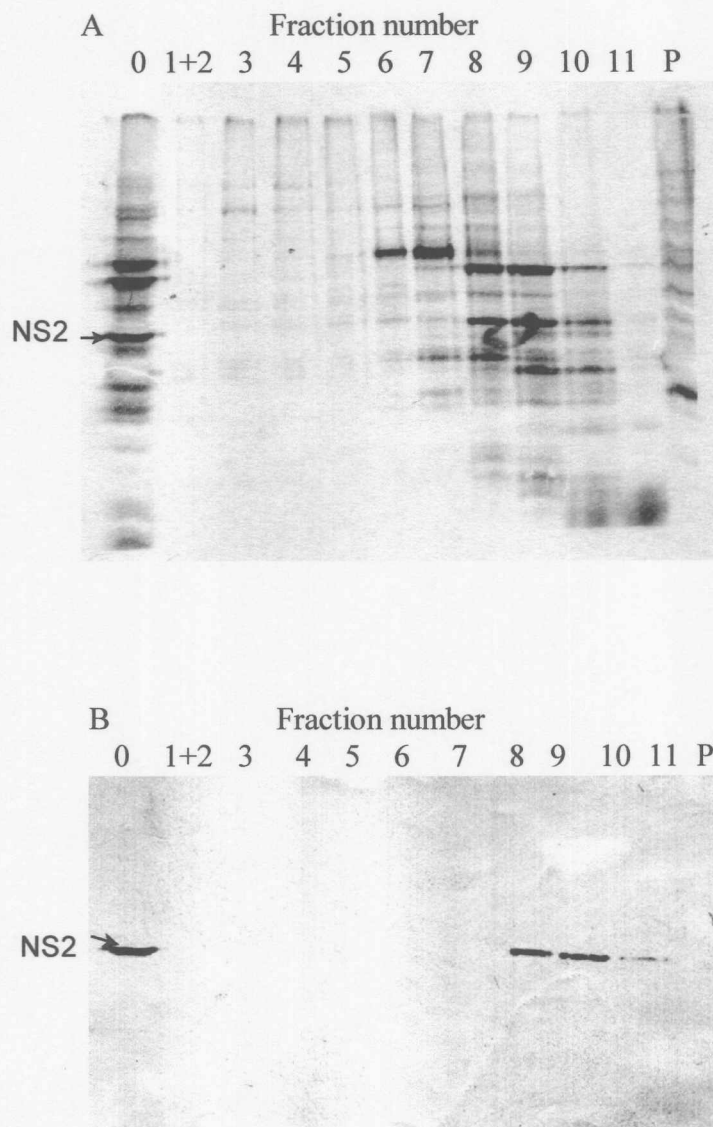
It is known that NS2 has an affinity for poly(U)-Agarose (Huisman *et al.*, 1987a). To determine how the chimeric NS2 compares with AHSV NS2 and BTV NS2 in its ability to bind ssRNA, a binding assay was performed by binding purified <sup>35</sup>S-labelled NS2 to poly(U)-Agarose in the presence of increasing concentrations of NaCl.

For this assay Sf9 cells were infected with all three different NS2 producing recombinant baculoviruses namely AcAHSV-9.8, AcBTV-10.8 and AcChimera. As described in section 2.2.16, the proteins in the cells were pulse-labelled from 30 h post infection until 33 h post infection with [<sup>35</sup>S]-methionine before they were harvested. Cytoplasmic extracts were prepared of the recombinant baculovirus-infected cells in 0.5 M STE-TX. Here a high salt concentration (0.5 M) was used as NS2 only binds ssRNA completely at low salt concentrations, at very high salt concentrations NS2 does not bind ssRNA at all (Uitenweerde *et al.*, 1995). NS2 derived from cytoplasmic extracts after treatment with 0.5 M NaCl would thus consist of unbound NS2 alone as it can not bind cellular ssRNA at those conditions. The cytoplasmic extracts were then analysed on 10-40% sucrose in 0.4 M STE-TX gradients as described in section 2.2.16 to prevent the NS2 from binding to any cellular ssRNA. After 16 h of centrifugation at 200 000 g, fractions were collected from the bottom of the gradient at 40 drops per fraction and analysed by SDS-PAGE. To determine which fractions consisted of the unbound NS2 proteins the gels were autoradiographed. Results were confirmed by Western blot analysis. In Fig. 2.9-2.11 both the autoradiographs (A) and

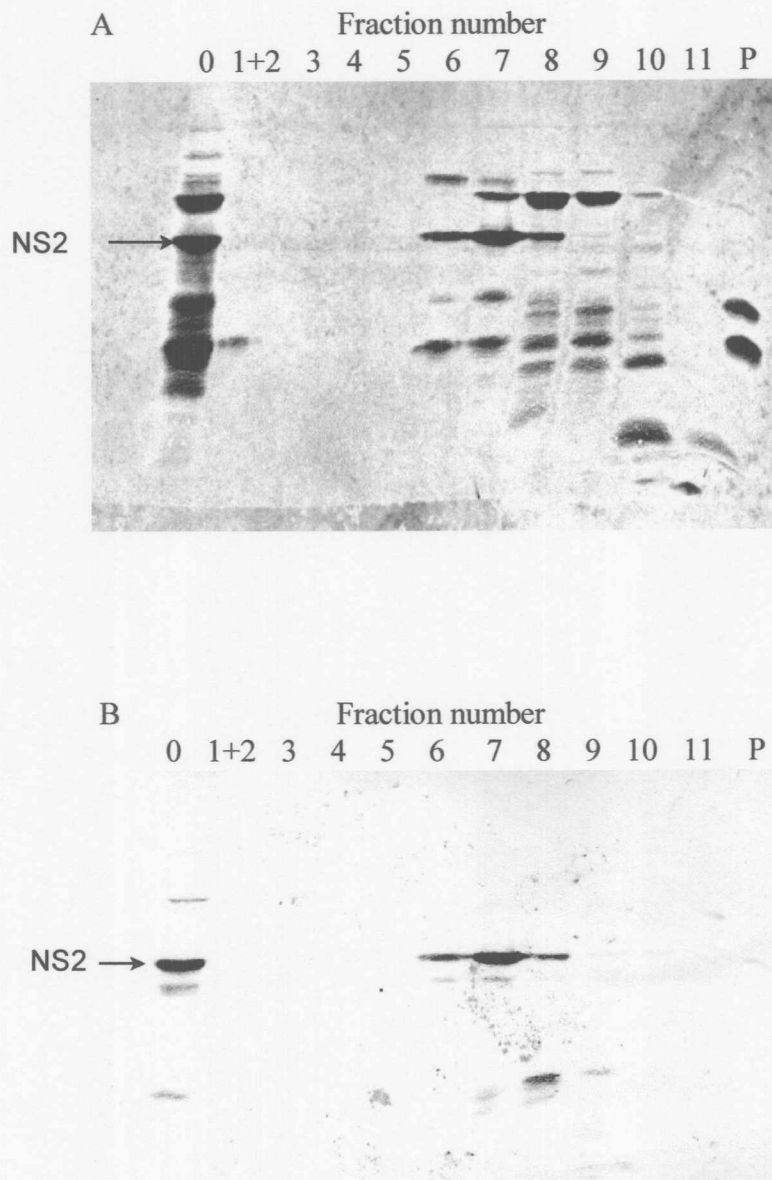
the Western blots (B) are shown.



**Fig. 2.9** (A) Autoradiograph and (B) Western-blot analysis, using rabbit  $\alpha$ AHSV NS2, of AcAHSV-9.8 infected Sf9 cells. Lanes (1 to 11) represent the fractions obtained from sucrose gradient centrifugation analysis of cytoplasmic extracts in 0.4 M NaCl from the infected Sf9 cells after [ $^{35}$ S]-methionine pulse labelling. Fraction 1 is derived from the bottom of the gradient and fraction 11 from the top. (0) is the unfractionated sample and (P) is the pellet. The position of NS2 is in each case indicated on the figure.



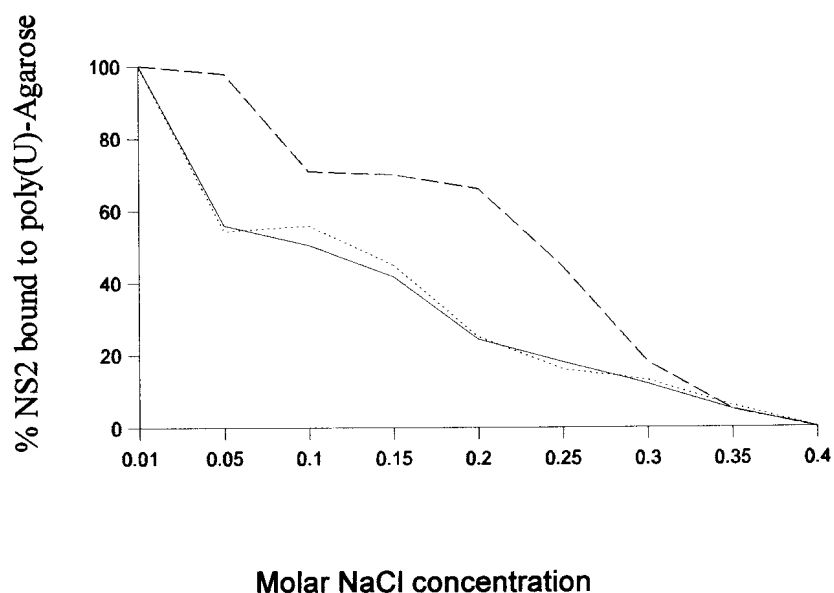
**Fig. 2.10** (A) Autoradiograph and (B) Western-blot analysis, using rabbit  $\alpha$ BTB NS2, of AcBTB-10.8 infected Sf9 cells. Lanes (1 to 11) represent the fractions obtained from sucrose gradient centrifugation analysis of cytoplasmic extracts in 0.4 M NaCl from the infected Sf9 cells after [ $^{35}$ S]-methionine pulse labelling. Fraction 1 is derived from the bottom of the gradient and fraction 11 from the top. (0) is the unfractionated sample and (P) is the pellet. The position of NS2 is in each case indicated on the figure.



**Fig. 2.11** (A) Autoradiograph and (B) Western-blot analysis, using rabbit  $\alpha$ AHSV NS2, of AcChimera infected Sf9 cells. Lanes (1 to 11) represent the fractions obtained from sucrose gradient centrifugation analysis of cytoplasmic extracts in 0.4 M NaCl from the infected Sf9 cells after [ $^{35}$ S]-methionine pulse labelling. Fraction 1 is derived from the bottom of the gradient and fraction 11 from the top. (0) is the unfractionated sample and (P) is the pellet. The position of NS2 is in each case indicated on the figure.

In each gradient the appropriate fractions containing the NS2 protein were pooled: In Fig. 2.9, fractions 5 and 6, in Fig. 2.10, fractions 8 and 9 and in Fig. 2.11, fractions 6 and 7. The fact that the proteins are spread over different fractions in the three different assays are due to a difference in drop size as well as a difference in the amount of drops per fraction due to a difference in drop speed as the protein distributions compared in previous attempts.

The poly(U)-Agarose binding assay was performed as described in section 2.2.16. After the cells were labelled with [<sup>35</sup>S]-methionine at 33 h post infection, harvested and washed, the cells were resuspended in 0.5 M STE-TX. The cells were then incubated, sonicated and the nuclei were removed by centrifugation. The supernatants were loaded on 10-40 % sucrose gradients in 0.4 M STE-TX and centrifugated for 16 h at 200 000 g to obtain unbound labelled NS2. Fractions were collected from the bottom of the gradient and the NS2 containing fractions were identified. The appropriate fractions were pooled and used to bind poly(U)-Agarose at nine different NaCl concentrations between 0.01 M and 0.4 M. Uitenweerde *et al.* (1995) showed that at a NaCl concentration of 0.01 M the NS2 proteins of different orbiviruses are quantitatively bound to poly(U)-Agarose. The assay was repeated for AHSV NS2, BTV NS2 as well as the chimeric NS2. The results obtained are shown in Fig. 2.12.



**Fig. 2.12** Graphic representation of the percentage of <sup>35</sup>S-labelled NS2 bound to poly(U)-Agarose at different NaCl concentrations. AcAHSV-9.8 NS2 (—), AcBTV-10.8 NS2 (---), AcChimere NS2 (.....).

The graph in Fig. 2.12 shows that all three types of NS2 proteins are completely bound to the poly(U)-Agarose at the lowest salt concentration of 0.01 M NaCl. BTV NS2 has a very high affinity for poly(U)-Agarose and stays almost completely bound at 0.05 M NaCl. It then drops to about 70% at 0.1 M NaCl and stays there up to 0.2 M NaCl. Only after 0.2 M NaCl does its binding ability decrease rapidly until it is not bound at all at about 0.35 M NaCl. AHSV NS2 on the other hand, does not bind so strongly to ssRNA as BTV NS2. There is an almost immediate decrease in binding ability to about 50% at 0.05 M NaCl. From the 0.1 M NaCl there is a gradual decrease in binding until it is not bound at all at about 0.35 M NaCl. The results obtained from the chimeric NS2 are almost identical to that of the AHSV NS2. This assay confirms the results obtained from the low salt sucrose gradient sedimentation analyses that show that the ssRNA binding affinity of the chimeric NS2 correlates with that of AHSV NS2 rather than with that of BTV NS2. The N-terminal half, rather than the  $\alpha$ -helixes seems to determine the strength of ssRNA binding.

## 2.4 DISCUSSION

The aim of this part of the study was to express the chimeric NS2 protein in the novel BAC-TO-BAC™ expression system in order to produce large amounts of the protein and to compare the ssRNA-binding ability of the chimeric NS2 with that of AHSV NS2 and BTV NS2 respectively. As the chimeric NS2 consists of the N-terminal region of AHSV NS2 and the  $\alpha$ -helix rich C-terminal region of BTV NS2 it could be used to determine to which degree the  $\alpha$ -helixes on the C-terminal are important for ssRNA binding.

In this study recombinant baculoviruses containing the chimeric NS2 gene were isolated with the first attempt. When the chimeric NS2 was compared with AHSV NS2 and BTV NS2 by SDS-PAGE it was found that the chimeric NS2 migrated at a lower rate than AHSV- and BTV NS2. All the NS2 proteins appear to migrate at a lower rate than their predicted 41 kDa, and migrate as proteins of about 46 kDa. Although the reason for this is unexplained, it was verified that the protein that migrated at a lower rate was indeed the chimeric NS2 and not a contaminant. This anomalous migration of NS2 make them analogous to the masking proteins that bind ssRNA for storage in oocytes, which is most likely the consequence of the modular distribution of charged residues on the proteins (Sommerville, 1992).

The chimeric NS2 was also shown to be soluble and able to bind ssRNA. When analysed by low salt sucrose gradient centrifugation the chimeric NS2 had the same sedimentation profile as AHSV NS2, and was easily distinguished from that of BTV NS2. The ssRNA binding affinity of the chimeric NS2 also compared to that of AHSV NS2 and not to that of BTV NS2 when their ability to bind ssRNA was compared in a poly(U)-Agarose binding assay. As the chimeric NS2 consists of the N-terminal of AHSV NS2 and the  $\alpha$ -helix rich C-terminal of BTV NS2, one would expect the binding ability of the chimeric NS2 protein to compare to that of BTV NS2 if the  $\alpha$ -helices were to play a major role in ssRNA binding as predicted. Either the  $\alpha$ -helices are not important in ssRNA binding in this protein and binding efficiency is mainly determined by the N-terminal portion, or the protein's three dimensional structure is so disturbed by the fact that it is a chimera between the proteins of two different viruses that it cannot bind properly to the ssRNA anymore.

In order to decide just how NS2 binds to ssRNA it is important to have an idea of how other ssRNA binding proteins bind.

The most widely found RNA-binding motif is the RNP motif. It is composed of 90 to 100 amino acids which form a RNA-binding domain that is present in one or more copies in proteins that bind pre-mRNA, mRNA, pre-ribosomal RNA and small nuclear RNAs. The identifying feature of the RNP motif is the RNP consensus sequence, which is composed of two short sequences, RNP1 'LFVGN' or 'IYIKGM' and RNP2 'KGFGFVXF' or 'RGYAFVXY' and a number of other, mostly hydrophobic conserved amino acids interspersed throughout the motif. Some RNA-binding proteins lack canonical RNP1 and RNP2 sequences but contain other well conserved structurally important residues so that the overall structure of these domains is very similar to RNP motif RNA-binding domains. The secondary structure of the RNP motif consists of  $\beta\alpha\beta\beta\alpha\beta$  and forms a four-stranded antiparallel  $\beta$  sheet packed against the two perpendicularly orientated  $\alpha$  helices. Amino acids of RNP1 and RNP2 are juxtaposed on the two central  $\beta$  strands of the folded domain. It is not likely that the highly conserved amino acids of RNP1 and RNP2, although crucial for RNA binding, distinguish between different RNA sequences. The major determinants of RNA-binding specificity reside rather in the most variable regions of the RNP motif, particularly in the loops and the termini. The  $\beta$  sheet of the RNA-binding domain, which contains many of the most highly conserved residues of the domain, constitutes just a general RNA binding surface to which distinct and variable determinants of specificity are added (Mattaj, 1993; Burd & Dreyfuss, 1994).



The Arginine-Rich Motif (ARM) consists of short (10 to 20 amino acids) arginine-rich sequences that mediate RNA binding. Other than the abundance of arginine residues most ARM proteins do not share a common structure. The RNA binding sites of ARM proteins are complex and consist of stem-loops, internal loops, or bulges, and their structure, rather than particular sequence, may be the major binding determinant. The positive charge of arginine increases nonspecific affinity for RNA, thereby facilitating the search for high-affinity binding sites. In this context the role of arginine may be to probe the local conformation of the RNA backbone, in search of the RNA determinants of high-affinity binding. Specific RNA binding by ARM peptides suggests that arginine-rich RNA-binding domains can bind RNA with specificity, shorter peptides however discriminate less well between specific and nonspecific RNAs compared with longer protein fragments (Mattaj, 1993; Burd & Dreyfuss, 1994). An example of a protein that binds to RNA with an ARM is the Nun protein of phage HK022. Nun is a 109-amino acid protein that is involved in transcription termination in bacteria (Watnick & Gottesman, 1999). Influenza A virus nonstructural protein 1 (NS1) uses a novel ARM to bind specifically to the polyA tails of mRNA. This binding ability resides on the N-terminal domain (residues 1-73) of the protein (Chien *et al.*, 1997; Marión *et al.*, 1997; Suarez & Perdue 1998).

The RGG box is a 20- to 25-amino acid long RNA binding motif typical found in combination with other types of RNA-binding domains. The motif is defined as closely spaced Arg-Gly-Gly (RGG) repeats interspersed with other, often aromatic, amino acids. RGG boxes usually occur in proteins that also contain other types of RNA-binding domains. RNA binding by the RGG box is relatively sequence nonspecific and it facilitates binding by one or more RNA-binding domains. Many RGG box-containing proteins contain the modified amino acid  $N^G$ ,  $N^G$ -dimethylarginine. Methylation would not affect the strong positive charge of the arginine side chain, but it could, by steric constraints, modulate RNA binding. Phosphorylation of adjacent residues is another potential mechanism for regulating RGG box RNA binding (Burd & Dreyfuss, 1994).

The K homology (KH) motif of several proteins is able to bind ssRNA *in vitro*, and a mutation (underlined) in the core sequence, VIGXXGXXI, of one of the two KH motifs encoded by the fragile X mental retardation gene (FMR-1) causes fragile X mental retardation. The three-dimensional structure of the KH motif is not yet known (Burd & Dreyfuss, 1994).

A small number of RNA-binding proteins, including retroviral nucleocapsid proteins, RNA polymerases and yeast RNA-binding proteins, contain sequences (appropriately spaced cysteine-histidine residues) that relate these proteins to the zinc finger family of DNA binding proteins. A generalised zinc finger-knuckle motif can be written as  $CX_{2-5}CX_{4-12}C/HX_{2-4}C/H$  (in which X represents any amino acid) (Burd & Dreyfuss, 1994).

Although the corresponding ssRNA-binding proteins of members of the *Reoviridae* family, namely NS2 in the orbiviruses (AHSV, BTV and EHDV),  $\sigma$ NS of reoviruses and NSP3 of rotaviruses show an extremely low level of sequence similarity, they do share a 9 amino acid consensus sequence ([I/L]XXM[I/L][S/T]XXG) in which five positions have identical or equivalent amino acids (Van Staden *et al.*, 1991). It was shown that if the aforementioned amino acid motif, amino acids 75-83, was deleted in NS2 of EHDV, the protein lost its ability to bind to ssRNA on a poly(U)-Sepharose column. With site-specific substitutions in this region it was shown that even a single substitution exhibited on average a 10-15% less affinity for the nucleic acid substrate than wild type NS2. When three or more amino acids were substituted, RNA-binding was almost completely abolished, similar to the deletion mutant (Theron *et al.*, 1996). According Theron *et al.* (1996) this region is important for the formation of the secondary and tertiary structure of NS2 necessary for ssRNA-binding rather than part of an RNA-binding domain since the sequence does not conform to one of the conserved motifs associated with RNA-binding proteins as reported by Burd & Dreyfuss, (1994). This domain is also predominantly hydrophobic with low surface probability and the residues would therefore not be available to form interactions with the nucleic bases. The deletion of this 9 amino acid motif did not alter the compartmentalisation of the protein and it was, like wild-type NS2, also present in the cytoplasm of infected cells. However, this motif does seem to be important for VIB formation as was evident by the lack of structural integrity of the bodies observed in mutant baculovirus-infected cells, compared to those in cells infected with the wild-type NS2 baculovirus recombinant (Theron *et al.*, 1996). Piron *et al.* (1999) also agree that this motif may be related to other functions rather than sequence specific RNA binding.

The question that has to be addressed is whether the ssRNA-binding affinity of the chimeric NS2, that consists of the  $\alpha$ -helix rich C terminal of BTV NS2 and the N-terminal of AHSV NS2, compares to that of AHSV NS2 rather than to that of BTV NS2 because of a disruption in the structural folding of the protein or because the binding domain resides on the N-terminal rather than on the

C-terminal of the protein.

To bind ssRNA with an  $\alpha$ -helical conformation is commonly known. Analysis of the secondary structure of segment 7 of Simian 11 rotavirus, encoding nonstructural protein NCVP4, also revealed the presence of a 160 amino acid region rich in potential  $\alpha$ -helix structure in the C-terminal region of the protein (Both *et al.*, 1984). In the case of influenza A virus it has been suggested that a region of the RNA binding matrix protein M1 could interact with a ribonucleoprotein particle by forming an  $\alpha$ -helix with lysine and arginine residues aligned on one face (Wakefield & Brownlee, 1989). Protein pr17, of the potato virus X, was shown to bind ssRNA in all cases. Computer analysis revealed the potential of the protein to form  $\alpha$ -helical structures within the acidic region. The second  $\alpha$ -helix would exhibit an amphipathic character with the negatively charged amino acid residues located predominantly on one side of the  $\alpha$ -helical wheel (Tacke *et al.*, 1991). The bacteriophage T4 RegA protein is a unique translational repressor that binds to the translation initiation region of target mRNAs. Its RNA binding domain were also mapped to an  $\alpha$ -helical domain (Gordon *et al.*, 1999).

It was suggested that a stretch of  $\alpha$ -helix containing positively charged amino acids is important for interaction with RNA in reovirus 3  $\sigma$ NS (Richardson & Furuichi, 1983). It is also interesting to note that a characteristic feature of  $\sigma$ NS that has been conserved in all three reovirus serotypes is the high content of  $\alpha$ -helix conformation; this includes a large  $\alpha$ -helix portion in the C-terminal, one-third of the molecule (Wiener & Joklik, 1987).

It is now known for reovirus nonstructural protein  $\sigma$ NS, a protein analogue of AHSV NS2, that the amino terminus and not the carboxy terminus is important for ssRNA binding as well as the formation of complexes larger than 7-9 S (Gillian & Nibert, 1998). According to predictions for protein secondary structure, the first 11-13 amino acids of the  $\sigma$ NS N-terminal form an amphipathic  $\alpha$ -helix, which is followed by a short turn region (Richardson & Furuichi, 1983). Thermolysin-mediated trimming of  $\sigma$ NS to generate the 41K form missing the first 11 residues occurs within a basic region near the C-terminal end of the predicted helix, suggesting that this region is flexible and exposed to solvent in the folded protein. Evidence that the deletion mutant is properly folded also suggests that the N-terminal region is exposed on the surface and not integral to the protein's overall structure. The predicted  $\alpha$ -helix itself may mediate important contacts so that when the

helical sequences are cleaved away from the protein,  $\sigma$ NS can no longer bind RNA, or the RNA contacts may be mediated by residues in the predicted turn region. In that case, thermolysin cleavage may not remove all the residues in  $\sigma$ NS that provide important contacts with RNA but may affect the local structure enough that RNA binding is disrupted (Gillian & Nibert, 1998).

Zhao *et al.* (1994), also indicated that the amino terminus of BTV NS2 is critical for RNA-protein interaction but not for oligomerisation. Certain amino acids located at the amino terminus, specifically residues within the first eight amino acids of the protein, play an important role in mediating NS2 binding to ssRNA i.e., the arginines at residues 6 and 7 and a lysine at residue 4, but not a glutamic acid at residue 2. Although these sequences are important, they do not rule out the possibility that other regions or sequences of NS2 cooperate in ssRNA binding. The carboxy terminus, including the last 130 amino acids, was found not to be required for ssRNA binding or for oligomerisation.

We have shown that the chimeric NS2 protein, which consists of the N-terminal of AHSV NS2, has the same ssRNA affinity as AHSV NS2. The  $\alpha$ -helices of the C-terminals of the different NS2 proteins therefore do not seem to play any role in ssRNA binding despite the correlation between the amount of  $\alpha$ -helices and the affinity for ssRNA between the proteins. The binding profile of AHSV is thus exclusively determined by the N-terminus, the region determined by amino acids 1-205. If the three-dimensional structure of the protein was affected it was in such a manner that it did not affect the ssRNA binding of the N-terminal region of the protein. Two deletion mutants of AHSV NS2 were also constructed in our laboratory. The first lacked three terminal amino acids and the second six amino acids, both at the N-terminal. When the deletion proteins were compared to AHSV NS2 for their ability to bind poly(U)-Agarose it was found that both mutants had a lower affinity for ssRNA than AHSV NS2. The mutant with the deletion of six amino acids also had a lower affinity for ssRNA than the mutant with the three amino acid deletion. This is further proof that the N-terminal and not the C-terminal is responsible for ssRNA binding. The first eight amino acids of AHSV NS2 also differ significantly from that of BTV NS2, there is only one arginine residue at position 5 and a lysine at position 6. The next arginine is only at position 12. The fact that AHSV NS2 has only one arginine residue in this region may be the reason why it has a lower ssRNA affinity than BTV NS2. NS2 does not have an analogue for any of the known ssRNA-binding motifs, this includes the RNP motif, ARM, RGG box, KH motif as well as zinc fingers.

There is thus no indication of how NS2 binds to ssRNA as the binding motif still eludes us. There is also no proof that NS2 can bind virus specific mRNA in a sequence specific manner. It is further also unclear where the ssRNA binding ability of NS2 fits into the life cycle of the virus. In the next chapter we will look at the binding of AHSV NS2 to virus specific mRNAs.

## CHAPTER 3

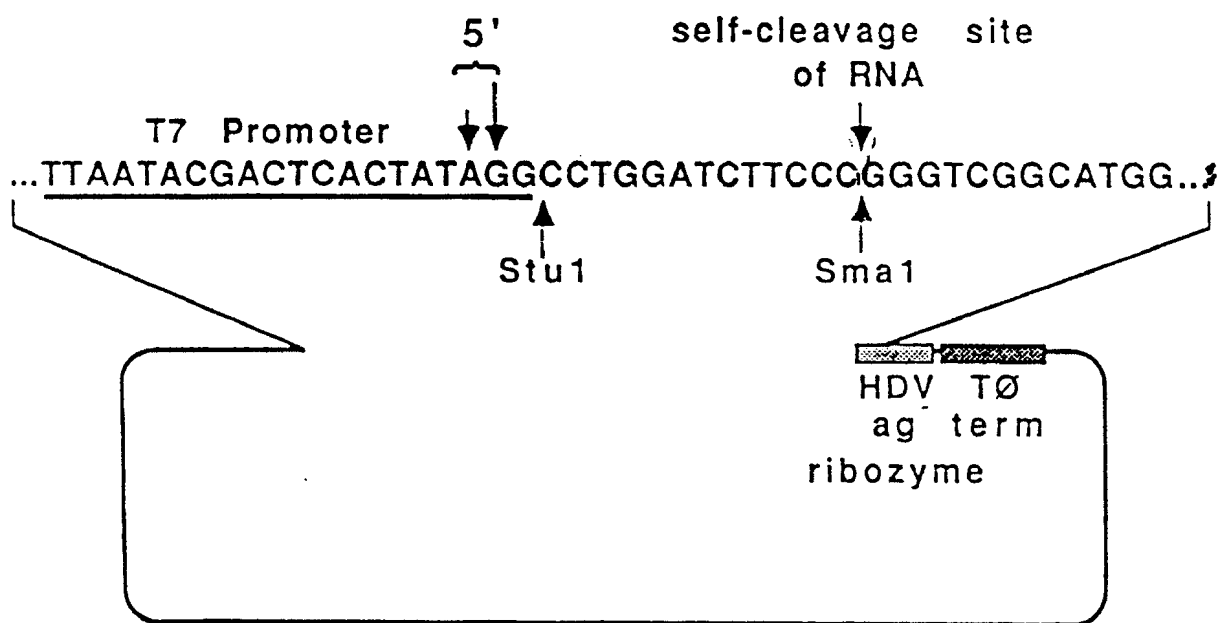
### THE CONSTRUCTION OF AHSV mRNAs WITH AUTHENTIC ENDS

#### 3.1 INTRODUCTION

The AHSV genome consists of 10 segments of dsRNA, each of which is transcribed into mRNA by a core-associated dsRNA-dependent polymerase (Van Staden *et al.*, 1991). Huismans *et al.* (1987b) identified the NS2 protein with an affinity for ssRNA and showed that BTV NS2 binds to BTV mRNA that was obtained by *in vitro* transcription of CsCl-purified BTV core particles. Only total mRNA content was used and not single authentic mRNA transcripts. It was then suggested that NS2 binds the virus specific mRNA for encapsidation. NS2 binds non-specifically to ssRNA (Thomas *et al.*, 1990) as can be seen in its binding to poly(U)-Agarose (Uitenweerde *et al.*, 1995). A question that comes to mind is whether AHSV NS2 has a specific affinity for individual AHSV mRNA strands. To answer this question we first needed some individual authentic AHSV mRNA strands. In order to obtain individual mRNA strands we wanted to use *in vitro* transcription (Theron & Nel, 1997). The only problem was that with normal *in vitro* transcription the products are not authentic mRNA's, as transcription is initiated upstream of the 5' end of the gene at the vector promoter and runoff transcription frequently occurs at the 3' end as transcription is terminated when the polymerase reaches the end of the linearised vector. Normally it does not matter whether the mRNAs' ends are authentic or not as they are only used for *in vitro* translation. In our case this was a real problem as NS2 has non-specific binding to ssRNA and we did not want binding to occur between the protein and the non-specific runoff parts of the transcripts. To overcome this problem of authentic ends we used transcription vector (2.0) (V(2.0)) which was kindly provided by L.A. Ball (Professor of Microbiology, The University of Alabama at Birmingham, Alabama).

V(2.0), as seen in Fig. 3.1, is a specially designed transcription vector that can be used to generate authentic mRNA segments. This system employed bacteriophage T7 DNA-dependent RNA polymerase to synthesise genomic RNA from circular transcription plasmids that were introduced into cells by transfection. The 5' end of the transcript was determined by the position of the T7 promoter relative to the viral insert, whereas the 3' end of the transcript was generated

by autolytic cleavage mediated by a ribozyme placed immediately downstream of the viral insert. The ribozyme thus cut the transcript at a precise point in the plasmid. As a PCR product, with authentic ends, was used and inserted into the specially designed site, the ribozyme ensured authentic mRNA transcripts without allowing any runoff transcription (Ball, 1992; Pattnaik *et al.*, 1992). To ensure authentic ends by correct self cleavage, the PCR product had to be inserted between the *Stu I* and *Sma I* sites, as seen in Fig. 3.1. As transcription initiation can start at either the A or the G upstream of the *Stu I* site, the virus specific mRNA will contain two or three extra nucleotides at the 5' end.



**Fig. 3.1** Transcription vector (2.0)

The aim of this part of the study was thus to construct recombinant transcription vectors that would provide us with individual mRNA transcripts of AHSV genes with authentic 3' and 5' ends. Some preliminary binding assays between the authentic mRNA transcripts and AHSV NS2 protein were also planned in order to determine if the protein has an affinity for the mRNAs with authentic ends.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Cells and plasmids

A variety of plasmids were used in this study:

(i) pAM25, a recombinant pUC13 plasmid containing a full length copy of the AHSV-3 S10 gene, coding for the NS3 protein, inserted into the *Bam HI* site of the vector, obtained from Mr. C.C. Smit (Department of Genetics, University of Pretoria).

(ii) p3.31, a recombinant pBS plasmid containing a full length copy of the AHSV-3 S9 gene, coding for the VP6 protein, inserted into the *Bam HI* site of the vector, obtained from Ms. P.J. de Waal (Department of Genetics, University of Pretoria).

(iii) pUC9.8, a recombinant pBS plasmid containing a full length copy of the AHSV-9 S8 gene, coding for the NS2 protein, inserted into the *Bam HI* site of the vector, obtained from Dr. V. van Staden (Department of Genetics, University of Pretoria).

(iv) pBRS7cDNA, a recombinant pBR plasmid containing a full length copy of the AHSV-9 S7 gene, coding for the VP7 protein, obtained from Mr. F.F. Maree (Department of Genetics, University of Pretoria).

(v) p9.8-V(2.0), a recombinant V(2.0) plasmid containing a full length copy of the AHSV-9 S8 gene in the T7 transcription promoter orientation, coding for the NS2 mRNA, obtained from Mr. F.T. Vreede (Biochemistry Division, Onderstepoort Veterinary Institute, Onderstepoort, South Africa).

(vi) Transcription vector (2.0) also called V(2.0), obtained from Prof. L. A. Ball. (Department of Microbiology, The University of Alabama at Birmingham).

All plasmids were propagated in XL1-Blue *E.coli* cells grown in LB-medium (1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl pH 7.4).



### 3.2.2 Polymerase chain reaction

PCR was utilised to construct full length gene fragments of the AHSV-3 S9 coding for VP6, AHSV-9 S7 coding for VP7 and AHSV-3 S10 coding for NS3. In each case the primers were constructed in such a way that the forward primer corresponds to the first few nucleotides of the gene and the reverse primer corresponds complementary to the last few nucleotides of the gene, without any overlap with the plasmid. In total, six primers were constructed and ordered from MWG-Biotech GmbH:

- (i) VP6.4, the forward primer for S9, 5' GTTAAATAAGTTGTCTCATG 3', with a  $T_m$  of 49.1°C.
- (ii) VP6.5, the reverse primer for S9, 5' GTAAGTTTTAAGTTGCCC 3', with a  $T_m$  of 49.1°C.
- (iii) VP7.A, the forward primer for S7, 5' GTTAAAATTCGGTTAGGATG 3', with a  $T_m$  of 51.2°C.
- (iv) VP7.B, the reverse primer for S7, 5' GTAAGTGATTTCGGTATTG 3', with a  $T_m$  of 50.2°C.
- (v) NS3I, the forward primer for S10, 5' GTTTAAATTATCCCTTGTCATG 3', with a  $T_m$  of 52.8°C.
- (vi) NS3II, the reverse primer for S10, 5' GTAAGTCGTTATCCCGGCTCC 3', with a  $T_m$  of 61.8°C.

Each PCR reaction mixture contained 25 ng template DNA (pAM25 or p3.31 or pBRS7cDNA), 100 pmol of each of the corresponding oligonucleotide primers (forward and reverse), 5  $\mu$ l of 10  $\times$  Taq polymerase buffer (500 mM KCl, 100 mM Tris.HCl (pH 9), 1% Triton X-100), 1.5 mM MgCl<sub>2</sub>, 6  $\mu$ l of a dNTP mixture (1.25 mM of each dNTP) and 1.5 U of Taq DNA polymerase (5 U/ $\mu$ l) (Promega). The volume was adjusted to 50  $\mu$ l with UHQ. The PCR reactions were conducted in a thermal cycler (MJ Research PTC-200 Peltier Thermal Cycler) under the following reaction conditions: The PCR process occurred in three parts. In the first part template denaturation was at 95°C for 5 min before primer annealing for 45 s at 45°C and primer extension and polymerisation at 72°C for 3 min. The second part was the main amplification step and consisted of 28 cycles. Each cycle consisted of a heat denaturation step of 1 min at 95°C followed by primer annealing at 45°C for 45 s and primer extension at 72°C for 3 min. The third and last part followed

then with a heat denaturation step of 1 min at 95°C, followed by primer annealing at 45°C for 45 s and primer extension at 72°C for 5 min.

### 3.2.3 Phosphorylation and Klenow

As the primers were constructed without 5' tri-phosphate groups, it was necessary to add these groups to the 5'-ends of the amplified PCR fragments in order to be able to clone it into a vector. Phosphorylation was performed by adding 5 µl of the 10 × buffer (50 mM Tris-HCl pH 7.2, 6 mM MgCl<sub>2</sub>, 10 mM DTT), 1 µl of ATP (100 nM) and 0.25 U polynucleotide kinase (Amersham) to 20 µl of the PCR reaction. The volume was adjusted to a final volume of 50 µl with UHQ and it was incubated at 37°C for 70 min. The enzyme was then denatured at 65°C for 3 min after which the fragments were analysed by agarose gel electrophoresis. The appropriate bands were excised from the gel and purified using the GeneClean™ method (section 2.2.7).

After phosphorylation and GeneClean™ purification, terminal nucleotides were added to the amplified PCR fragments to fill in the overhangs and generate blunt-ends by using Klenow DNA Polymerase I enzyme (Boehringer Mannheim). 0.5 U of enzyme was added to the 50 µl phosphorylation reaction mixture and the mixture was incubated for 30 min at 37°C. The enzyme was denatured by heating at 65°C for 3 min and the fragments were again analysed by gel electrophoresis, excised from the gel and purified using the GeneClean™ method.

### 3.2.4 Blunt-end ligation

To a mixture containing purified vector and insert (in a ratio of 1:10), 1 U T4 DNA ligase (Boehringer Mannheim) was added together with 2 µl T4 ligase buffer (66 mM Tris.HCl (pH 7.5), 5 mM MgCl<sub>2</sub>, 1 mM DTT, 1 mM ATP) in a final reaction volume of 20 µl. This mixture was incubated at 20°C for 16 h.

### 3.2.5 Sequencing

To ensure that the template DNA was of optimal quality for sequencing it was necessary to purify the recombinant V(2.0) vectors containing the desired fragments. The Qiagen<sup>R</sup> Plasmid mini-kit

was used for template purification. All sequencing reactions were done by cycle sequencing and the manufacturer's instructions were followed at all times. First the genes that were to be sequenced had to be amplified. This amplification was done by PCR using four different dye-labelled ddNTP- terminators of different colours, one for each of the four different dNTP's. The enzyme used for the PCR reactions was AmpliTaq<sup>R</sup> DNA Polymerase FS. This enzyme is a mutant form of *Taq* DNA polymerase and has almost no 5'-3' nuclease activity. As AmpliTaq<sup>R</sup> DNA Polymerase FS has a drastic increase in its discrimination against the incorporation of ddNTPs when compared to other *Taq* polymerases, high concentrations of dye-terminators had to be used. Both the dye-terminators and the AmpliTaq<sup>R</sup> DNA Polymerase FS were supplied in the ABI PRISM<sup>TM</sup> Dye Terminator Cycle Sequencing Ready Reaction kit. Each PCR reaction consisted of 3.2 pmole of the V(2,0) forward and reverse primers (T7rec, the V(2.0) forward primer, 5' GGAAGCGGAAGAGCGCCC 3', with a T<sub>m</sub> of 62°C and T7term, the V(2.0) reverse primer, 5' GCCAACTCAGCTTCCTTTCGGG 3', with a T<sub>m</sub> of 70°C), together with 8 µl of the included Terminator Ready Reaction Mix and 300 ng of the double-stranded template. The reaction volume was then made up to 20 µl with UHQ. PCR was performed in a MJ Research PTC-200 Peltier Thermal Cycler DNA Engine with a heated lid. The PCR amplification process consisted of 25 cycles. Each cycle consisted of a rapid thermal ramp to 96°C where the ddDNA templates were allowed to denature for 10 s, a rapid cooling ramp to 50°C for annealing and a rapid thermal ramp to 60°C where primer extension occurred for 4 min. After the PCR process the excess dye terminators were removed by ethanol precipitation. In each case 2 µl 3 M sodium acetate (pH 4.6) was added to 50 µl 95% EtOH before it was again added to a 20 µl sample of the PCR reaction. The reaction was then vortexed, placed on ice for 10 min and centrifuged in a microfuge for 15 min. The pellet was rinsed with 70% EtOH, repelleted and vacuum dried. Samples were then resuspended in 25 µl Template Suppression Agent, vortexed and the DNA precipitated by centrifugation. The DNA was then denatured for 2 min at 95°C, mixed by vortexing and the DNA was concentrated by centrifugation before it was placed on ice. The samples were loaded in an ABI PRISM<sup>TM</sup> 310 Analyzer as it was used for sequencing. Sequences were analysed using the ABI PRISM Sequencing Analysis<sup>TM</sup> program, as well as the ABI PRISM Navigator<sup>TM</sup> program.

### 2.3.6 Phenol/chloroform extraction

In order to perform successful *in vitro* transcription reactions it was necessary to have the template

DNA as clean as possible. Phenol/chloroform extractions were thus performed on the recombinant plasmid DNA, obtained from large scale plasmid purifications, in order to remove all the contaminating proteins. 400 µl of the large scale reactions were used to which equal volumes of a phenol:chloroform:isoamyl alcohol mixture (25:24:1) were added. Everything was mixed gently before it was centrifuged at 17 000 g for 5 min. During the centrifugation process the organic and aqueous phases separated and the upper aqueous phases were recovered. The aqueous phases were extracted twice more with equal volumes of chloroform. The DNA was then precipitated by the addition of 2.5 volumes 96% ethanol and a final concentration of 0.3 M Na-acetate (pH 7.0). After centrifugation for 10 min at 17 000 g, the DNA was washed with 70% ethanol and vacuum dried. At the end 400 µl of DEPC-treated UHQ was again used to resuspend the DNA pellets.

### 3.2.7 *In vitro* transcription

All *in vitro* transcription reactions were performed as described in: Protocols and Applications Guide, Promega. Each reaction mixture contained 1-2 µg of the template DNA, 5 µl of the 10 × transcription buffer (50 mM Tris.HCl (pH 7.6), 10 mM MgCl<sub>2</sub>, 10 mM 2-mercapto-ethanol), 2.5 µl of each rNTP (10 mM), 20 U of human placental RNase inhibitor (101 U/CII) and 19 U of T7 RNA polymerase (19 U/µl). The reaction volumes were adjusted to 50 µl with DEPC-treated UHQ and incubated at 37°C in an MJ Research PTC-200 Peltier Thermal Cycler DNA Engine without the heated lid. After 1.5 h 25 µl of each reaction was analysed by agarose gel electrophoresis. The electrophoretic equipment was soaked in a 10% NaOH-solution, rinsed with 70% ethanol and rinsed again in DEPC-treated UHQ prior to use in order to make sure that no RNase contamination would occur.

In order to obtain tritium (<sup>3</sup>H)-labelled mRNA, 2 µl 1.0 mCi/ml [5,6-<sup>3</sup>H]Uridine 5'-triphosphate, ammonium salt (Amersham) was first incubated for 10 min at -70°C and then freeze-dried for 20 min to evaporate the ethanol and water in which it was received. The reaction mixture, as described above, was then added to the [5,6-<sup>3</sup>H]Uridine with the only exception that this time only 2 µl of the cold UTP was used instead of 2.5 µl. The rest of the transcription process followed as described above.

### 3.2.8 Preparation of radiolabelled dsDNA probes

Probes were prepared from S7, 8, 9 and 10-specific DNA fragments. S8, 9 and 10 DNA fragments were excised by *Bam HI* restriction of the recombinant plasmids pUC9.8, p3.31 and pAM25. S7 fragments were excised from the recombinant plasmids pBRS7cDNA by *Eco RI* and *XbaI* restriction. The enzyme reactions were set up and were incubated at 37°C for 1 h (section 2.2.6). The samples were then run on an agarose gel and the required fragments were purified using the GeneClean™ method. After the fragments were purified, they were labelled by nick translation. In order to perform the nick translation reactions a Promega nick translation kit was used. Each reaction contained 200-500 ng of DNA, 2.5 µl of the nick translation 10× buffer (500 mM Tris.HCl (pH 7.2), 100 mM MgSO<sub>4</sub>, 1 mM DTT), 4 µl of a dNTP mixture (0.3 mM of each dATP, dGTP and dTTP), 10 µCi [ $\alpha$ -<sup>32</sup>P]dCTP and 2.5 µl of the enzyme mix. The reaction volumes were adjusted to 20 µl with UHQ and the reaction mixtures were incubated at 15°C. After 1 h the reaction was stopped by the addition of 5 µl stop solution (250 mM EDTA (pH 8.0)).

Labelled DNA was separated from the unincorporated radionucleotides by Sephadex column chromatography. Sephadex G75 beads were swollen overnight in DEPC-treated UHQ before they were autoclaved. A small glass bead was placed in a 5 ml Pasteur pipette and the swollen Sephadex G75 was poured into the pipette and allowed to settle until the level was about 1 cm from the top. The columns were then rinsed with 3 ml wash buffer (1 × TE, 0.5% SDS). The nick translation reaction mixtures were placed on top of the columns after the volumes were adjusted to 100 µl with DEPC-treated UHQ. This was followed by 500 µl of wash buffer and 600 µl fractions were collected from the bottom of the columns. The columns were then washed with another 10 × 100 µl wash buffer and another 10 fractions were collected of 100 µl each. All 11 fractions from each column were counted in a liquid scintillation counter (Beckman Model LS 3801) and the fractions containing the first peak by radioactivity were pooled and used as probes.

### 3.2.9 Northern blot analysis

A 1% agarose gel was prepared by adding the agarose to DEPC-treated UHQ in an Erlenmeyer flask and boiled. The agarose was allowed to cool down to 60°C, formaldehyde was added to a final concentration of 7.2% and 20 × MOPS buffer (0.4 M N(Morpholino)propanesulfonic acid (MOPS), 100 mM NaAc, 20mM EDTA, pH 7). After the gel had set, the gel chamber was filled with

1 × MOPS buffer without submerging the gel and the gel was covered with plastic wrap. The transcription reactions (section 3.2.7) were denatured, 3µl sample in 9µl loading buffer (50% formamide, 7.2% formaldehyde, 1 × MOPS buffer, X-cyanol), for 10 min at 68°C. The samples were then loaded on the gel and were subjected to electrophoresis at 100 V, 25 mA for 2 h.

The Northern blot analysis was done as a variation of the Southern blot analysis (Southern, 1975). First the gel was incubated for 30 min while it was gently shaking in 20 × SSPE (3.6 M NaCl, 2 mM Na<sub>2</sub>EDTA, 0.2M each of NaH<sub>2</sub>PO<sub>4</sub> and Na<sub>2</sub>HPO<sub>4</sub> pH 7). The blotting setup was prepared by stacking six pieces of filter paper, soaked in 20 × SSPE, on a piece of Gladwrap. The agarose gel was then placed onto the filter papers and all the air bubbles were removed. Gladwrap was then folded tightly against the sides of the gel to seal the filter paper/gel-sandwich properly. A Hybond™ N nylon membrane, the same size as the gel, was then layed on top of the gel followed by four more pieces of filter paper. Both the membrane and the filter paper were soaked in 2 × SSPE and all air bubbles were once again removed. Before the membrane was placed on top of the gel, the orientation of the membrane was marked by cutting off matching corners of both the gel and membrane. Lastly a 10 cm stack of paper towels were placed on top of the wet filter papers and weighed down by a light weight. The stack was left alone and transfer occurred by osmosis. The filter was removed after 18 h and air dried. The mRNAs were then fixed to the membranes by UV irradiation for 5 min on each side.

The membrane was then sealed in a plastic bag together with prehybridisation buffer (50% formamide, 5 × SSPE, 0.1% SDS, 0.1% Denhardt's solution (5% BSA, 5% Ficoll, 5% polyvinyl pyrrolidone), 5 mg salmon sperm DNA) and prehybridised at 42°C with shaking. After 1 h the prehybridisation buffer was removed and replaced with hybridisation buffer. The prehybridisation and hybridisation buffers were identical. The denatured probe (section 3.2.8) was also added to the membrane in hybridisation buffer and hybridisation was allowed to proceed at 42°C with shaking. The membrane was removed after 24 h and was washed in 2 × SSPE for 15 min at 42°C. This was followed by another two washes in 2 × SSPE, 1% SDS at 55°C for 15 min and again twice in 0.2 × SSPE for 15 min at 60°C, for stringency purposes. After the washes the membrane was air dried and exposed to X-ray film, overnight at -70°C, in a cassette with intensifying screens .

### 3.2.10 Purification of mRNA fragments from agarose gels

mRNA fragments were purified from agarose gel slices with The RNaid<sup>R</sup> KIT (BIO 101 Inc.), according to the manufacturer's instructions. 25 µl of the transcription reaction was loaded per well and the gel was run at 120 V and 100 mA for 40 min. The mRNA fragments were excised from the gel, mixed with 3 volumes of the RNA Binding Salt provided and incubated at room temperature for 10 min to dissolve the agarose. The RNAMATRIX<sup>R</sup> was added (6 µl) along with 10 µl 10% acetic acid and the mRNA was allowed to bind at room temperature for 5 min. The mRNA-RNAMATRIX<sup>R</sup> complex was pelleted by centrifugation for 1 min and the pellet was washed 3× RNA Wash. The purified mRNA was eluted from the RNAMATRIX<sup>R</sup> at 50°C in a final volume of 20 µl DEPC treated UHQ. The procedure was repeated twice, as each transcription reaction consisted of 50 µl, and the two volumes were pooled to give a total volume of 40 µl.

### 3.2.11 mRNA binding assay

$1 \times 10^7$  Sf9 cells were infected with the respective NS2 recombinant baculoviruses at a multiplicity of 40 pfu/cell infection for this assay. [<sup>35</sup>S]-methionine was added at 30 h postinfection (section 2.2.13) in order to label the proteins. After three hours of incubation the cells were washed twice in 1×PBS before they were resuspended in 500 µl 0.5 M STE-TX (0.5 M NaCl, 10 mM Tris, pH7.4, 1 mM EDTA, 0.5% Triton X-100). The cells were then incubated for 30 min on ice after which they were sonicated. The nuclei were then spun down for 5 min at 1 000 g in a microcentrifuge. The supernatants were removed and loaded on top of 10-40% sucrose gradients in 0.4 M STE-TX. The gradients were then centrifuged for 16 h at 200 000 g (SW50 rotor) to obtain unbound NS2. Forty drops per fraction were collected from the bottom of each gradient and the fractions containing the 7S NS2 multimer were identified by SDS-PAGE (section 2.2.12) and autoradiography (section 2.2.13). These fractions were pooled and used to assay RNA-binding. A quantity of the pooled sucrose gradient fractions (10 µl) was added to 40 µl purified H<sup>3</sup>-labelled mRNA, at a NaCl concentration of 0.01 M in a final volume of 50 µl. The proteins were allowed to bind to the mRNA at 4°C for 30 min with gentle agitation. The 50 µl fractions containing the bound NS2-mRNA complexes were loaded on 10-40% sucrose gradients in 0.15 M STE and centrifuged for 4.5 h at 325 000 g (SW50 rotor). Five drops per fraction were collected from the bottom of the gradients and were quantitated by scintillation counting in a liquid scintillation counter (Beckman Model LS

3801). There was distinguished between the H<sup>3</sup>-labelled mRNA and the S<sup>35</sup>-labelled NS2 proteins by using different windows to count the two radio-isotopes. The H<sup>3</sup>-labelled mRNA were counted in the window from 0 to 400 and the S<sup>35</sup>-labelled NS2 proteins were counted in the window from 400 to 700.

### 3.3 RESULTS

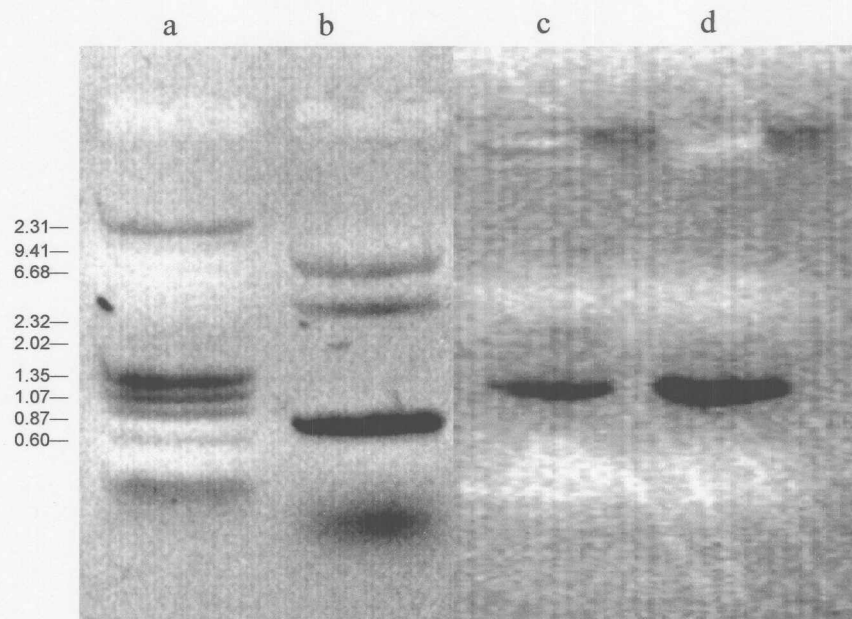
The results observed in this part of this study relate to the insertion of the VP6, VP7 and NS3 genes of AHSV into transcription vector V(2.0). The presence of the genes were confirmed by sequencing and the recombinant V(2.0) vectors were transcribed in order to obtain mRNA with authentic ends of the NS2, NS3, VP6 and VP7 genes of AHSV. The mRNAs obtained from the transcribed genes were also analysed by Northern blot analysis. Results of some very preliminary binding assays between the purified mRNA and AHSV NS2 are also described.

#### 3.3.1 Insertion of full length fragments of AHSV S7, S9 and S10 into transcription vector V(2.0)

The vectors containing copies of the S7, S9 and S10 genes of AHSV, pPBR7cDNA, p3.31 and pAM25 respectively (section 3.2.1), were obtained by large scale plasmid isolation (section 2.2.4). PCR was used to selectively amplify these gene fragments, using primers that have sequences based on the plus and minus strands respectively of the template. The primers were designed in such a way that after amplification of the full length PCR fragments the ends correspond exactly to the ends of the respective genes. The different primers that were used, as well as the PCR conditions have been discussed in section 3.2.2. The PCR products were then analysed by agarose gel electrophoresis (section 2.2.5). From Fig. 3.2 it can be seen that in each case a single band was amplified from a circular template. Only in lane (b) there was more than one band. The top two bands are open and close circle pAM25 and the third band from the top is the PCR product. The PCR product of S9 in lane (c) is 1169 bp long and migrates at the same rate as the 1167 bp PCR product of S7 in lane (d). All three fragments were of the expected sizes when compared to the size markers.



The PCR fragments were then phosphorylated at the 5' ends, as the primers were constructed without 5' tri-phosphate groups (section 3.2.3). After phosphorylation the fragments were GeneClean™ purified (section 2.2.7). The overhangs were then filled in using the Klenow enzyme (section 3.2.3) to ensure that the fragments were blunt ended. The fragments were again GeneClean™ purified to get rid of any contaminants that could interfere with the blunt-end ligation.



**Fig. 3.2** Agarose gel (1%) showing PCR amplification reactions used to generate three different gene fragments. Lane (a) shows size markers phiX and SMII. Lane (b) represents the PCR product of S10, lane (c) that of S9 and in lane (d) that of S7. S9 and S7 PCR products serve as size controls to each other.

The transcription vector, V(2.0), was digested with *SmaI* and *StuI* restriction enzymes in the appropriate buffers. Insertion between the *SmaI* and *StuI* restriction enzyme sites was necessary to ensure that the genes were at the correct distance from the promoter and also to ensure that the ribozyme would cut the mRNA at the correct position. The vector was first digested with *SmaI* (2h at 37°C) and then by *StuI* (2h at 37°C). Simultaneous digestion with the two enzymes resulted in incomplete *SmaI* digestion. The linearised vector was then dephosphorylated (section 2.2.8) to prevent self ligation. The linearised vector and the PCR products were then blunt-end ligated, with

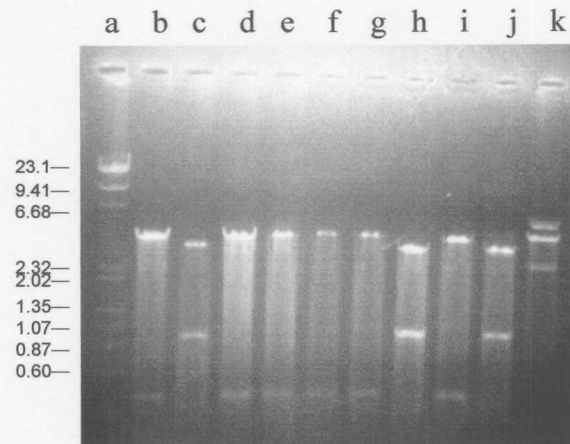
a vector to insert ratio of 1:10, at 20°C for 16 h (section 3.2.4).

*E. coli* XL1-Blue cells were made competent (section 2.2.2) and were transfected with the ligation mixture (section 2.2.3). The transformed cells were then plated out onto LB-agar plates containing Ampicillin and Tetracycline and incubated overnight. As vector V(2.0) has Ampicillin resistance, only bacterial colonies containing the vector will grow. Although a selection system is present to distinguish between bacterial colonies with and without the plasmid, there is no selection system to distinguish between recombinant vectors and vectors that had self-ligated. A large number of colonies were thus selected and analysed by mini-prep plasmid extractions and agarose gel electrophoresis. Samples with a lower rate of migration were selected (not shown). As there are no restriction sites to regain the cloned fragments from vector V(2.0), it was impossible to determine if the cloned fragments were of the right size by cutting it out of the vector and estimating its size. The only way to determine whether the cloned fragments were the correct fragments was by restriction enzyme orientation determination for each of the three different ligations.

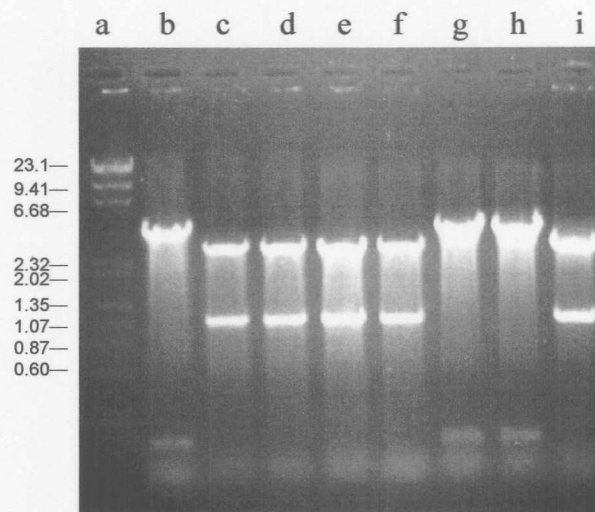
For the S9 cloning it was determined that the 3086 bp vector V(2.0) has a *Bam* *HI* site, 100 bp from the *Sma* *I* restriction site. S9 has an *Eco* *RI* site at 881 bp that divides the 1169 bp gene into a 881 bp and a 288 bp fragment. Digestion of the recombinant vector containing S9 with both *Eco* *RI* and *Bam* *HI*, results in a 388 bp fragment if the gene is in the right orientation leaving a bigger fragment of 3900 bp. If cloned in the wrong orientation a 981 bp fragment will be excised leaving a bigger 3300 bp fragment. In Fig. 3.3 it can be seen that lanes (b, d, e, f, g and i) consist of recombinant vector with S9 in the right orientation, while lanes (c, h and j) contain recombinant vector with S9 in the wrong orientation. The isolate in lane (k) probably consists of vector with two inserts.

For the S7 cloning it was determined that the 3086 bp vector V(2.0) has a *Bam* *HI* site 100 bp from the *Sma* *I* site into which gene fragments were inserted. The S7 gene has a *Bam* *HI* site at 100 bp that divides the 1167 bp gene into a 100 bp and a 1067 bp fragment. If the recombinant vector containing S7 is cut with *Bam* *HI*, a 1200 bp fragment will be cut out if the gene is in the right orientation leaving a bigger fragment of 3086 bp, or a 200 bp fragment will be cut out if the gene is in the wrong orientation leaving a bigger 4086 bp fragment. In Fig. 3.4 it can be seen that lanes (c, d, e, f and i) consist of recombinant vector with S7 in the right orientation, while lanes (b, g and

h) consist of recombinant vector with S7 in the wrong orientation.



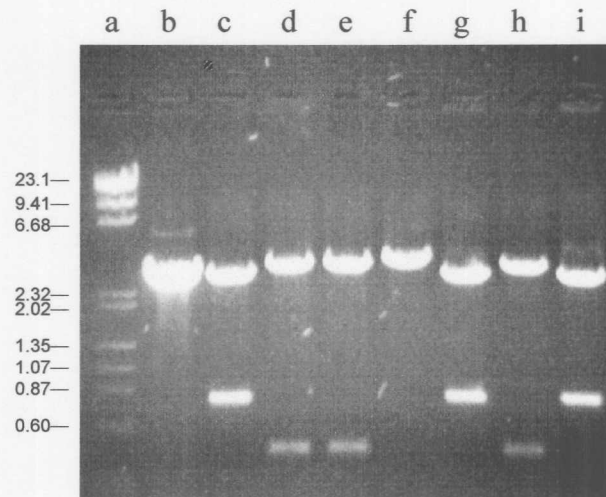
**Fig. 3.3.** Lane (a) contains size markers with sizes as indicated. Lanes (b-k) all show V(2.0) recombinants containing S9 genes that were digested with *Eco RI* and *Bam HI* for orientation determination.



**Fig. 3.4.** Lane (a) contains size markers with sizes as indicated. Lanes (b-i) all show V(2.0) recombinants containing S7 genes that were digested with *Bam HI* for orientation determination.

*Hind III* and *Bgl II* were used for orientation determination in the case of the S10 cloning. The 3086 bp vector V(2.0) has a *Hind III* site 273 bp from the *SmaI* site into which the gene fragments were inserted. S10 does not have a *Hind III* site, but it does have a *Bgl II* site at 553 bp. Digestion of the

758 bp gene with *Bgl II* produces a 553 bp and a 205 bp fragment. If the recombinant vector containing S10 is cut with both *Hind III* and *Bgl II*, a 478 bp fragment is excised if the gene is in the right orientation leaving a bigger fragment of 3366 bp, or a 826 bp fragment will be excised if the gene is in the wrong orientation leaving a bigger 3018 bp fragment. Fig. 3.5 represents recombinant vector with S10 in the right orientation (lanes d, e and h), and recombinant vector with S10 in the wrong orientation (lanes c, g and i).



**Fig. 3.5.** Lane (a) contains size markers with sizes as indicated. Lane (b) represents vector V(2.0). Lanes (c - i) all show V(2.0) recombinants containing S10 genes that were digested with *Hind III* and *Bgl II* for orientation determination.

### 3.3.2 Sequencing of the 5' and 3' ends of the genes

In order to verify that the recombinant vectors contained full length copies of the different genes, it was decided to sequence some of the clones. Three clones containing the S9 gene were chosen, two in the correct orientation (lanes d and e in Fig. 3.3, subsequently designated pV(2.0)VP6-3 and pV(2.0)VP6-4) and one in the wrong orientation (lane h in Fig. 3.3, subsequently designated pV(2.0)VP6-10). In the case of the clones containing S7, four clones were chosen. Three clones in the correct orientation (lanes c, d and f in Fig. 3.4, subsequently designated pV(2.0)VP7-2, pV(2.0)VP7-3 and V(2.0)VP7-5) and one in the wrong orientation (lane b in Fig. 3.4, subsequently designated pV(2.0)VP7-1) were chosen. Three clones containing the S10 gene were



also chosen, again two in the co and e in Fig. 3.5, subsequently designated pV(2.0)NS3-2 and pV(2.0)NS3-3) and one in the wrong orientation (lane c in Fig. 3.5, subsequently designated pV(2.0)NS3-1. All ten chosen clones were subjected to cycle-sequencing using the V(2.0) forward and reverse sequencing primers as described in section 3.2.5. This was also necessary to determine if any misincorporation of nucleotides had occurred due to the PCR reaction itself. Following sequencing the obtained sequences were compared with their known sequences as all three genes had previously been sequenced: AHSV-3 S10 by Van Staden *et al.* (1991), AHSV-3 S9 by Turnbull *et al.* (1996) and AHSV-9 S7 by Maree *et al.* (1998a). The sequencing results were as follows:

pV(2.0)VP6-3: Full length S9 clone in the correct orientation.  
pV(2.0)VP6-4: Full length S9 clone in the correct orientation.  
pV(2.0)VP6-10: Full length S9 clone in the wrong orientation.

pV(2.0)VP7-1: Full length S7 clone in the wrong orientation.  
pV(2.0)VP7-2: Full length S7 clone in the correct orientation.  
pV(2.0)VP7-3: Full length S7 clone in the correct orientation.  
pV(2.0)VP7-5: S7 clone in the correct orientation, but lacking the first two basepairs (G and T) on the 5' end.

pV(2.0)NS3-1: S10 clone in the wrong orientation, but lacking the last twelve basepairs (GTTTAAATTACC) on the 3' end.  
pV(2.0)NS3-2: Full length S10 clone in the correct orientation.  
pV(2.0)NS3-3: S10 clone in the correct orientation, but lacking the last three basepairs (GTA) on the 3' end.

### 3.3.3 *In vitro* transcription and Northern blot analysis

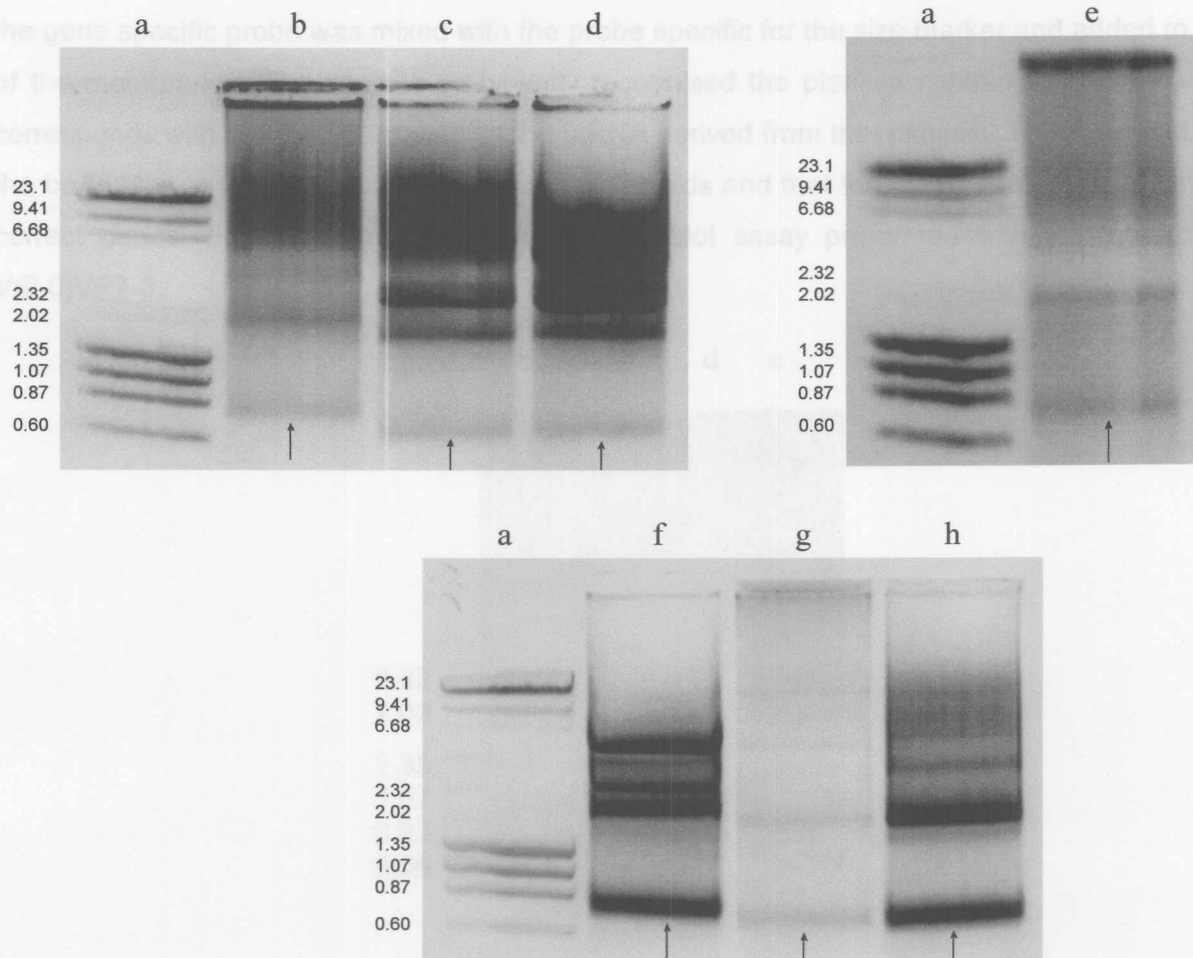
*In vitro* transcription was performed on several of the recombinant plasmids obtained in section 3.3.2 as well as on p9.8-V(2.0) in order to obtain mRNA of the different AHSV genes according to the method described in section 3.2.7. mRNA was obtained and was analysed electrophoretically. The different ribozyme cleaved mRNA fragments can be seen in Fig. 3.6

between the 0.87 kDa and the 0.60 kDa markers. The mRNA fragments seem smaller when compared to the corresponding genes in Fig. 3.2. This is due to the fact that the markers used are dsDNA and not ssRNA. The other bands consist of the linearised-, open circle and supercoiled closed circle plasmids as well as a band that contains mRNA of the whole plasmid as the ribozyme does not always cut.

mRNA derived from p9.8-V(2.0), pV(2.0)NS3-2, pV(2.0)VP6-3 and pV(2.0)VP7-3 was used in the assays. In each case mRNA derived from all four sources were run next to each other in each of four different gels and were then blotted to four different membranes. In each case

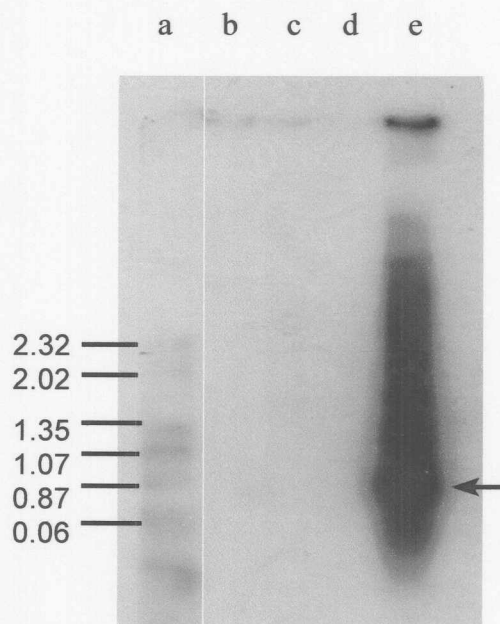
the gene specific probes were mixed with the probes specific for the ribozyme. The probes of the

conserved regions of the genome were used to detect the presence of the ribozyme. The probes



**Fig. 3.6** Agarose gels showing mRNA obtained by in vitro transcription. In each case lane (a) contains size markers with sizes as indicated. Lanes (b - h) show the products of the *in vitro* transcription reactions from p9.8-V(2.0) in lane (b), pV(2.0)NS3-2 in lane (c), pV(2.0)NS3-3 in lane (d), pV(2.0)VP6-3 in lane (e), pV(2.0)VP7-1 in lane (f), pV(2.0)VP7-3 in lane (g) and pV(2.0)VP7-5 in lane (h). The arrows indicate the positions of the different mRNAs.

In order to verify whether the fragments were really mRNA derived from the specific genes Northern-blot assays were performed. Four different  $^{32}\text{P}$ -probes were prepared, one each for detection of the NS2, NS3, VP6 and VP7 genes, as described in section 3.2.8 as well as a probe for detection of the size marker. The Northern-blot assays were then performed as described in section 3.2.9. Only mRNA derived from p9.8-V(2.0), pV(2.0)NS3-2, pV(2.0)VP6-3 and pV(2.0)VP7-3 was used in the assays. In each case mRNA derived from all four sources were run next to each other in each of four different gels and were then blotted to four different membranes. In each case the gene specific probe was mixed with the probe specific for the size marker and added to one of the membranes. The specific probe only recognised the plasmid containing the gene that corresponds with that probe, as well as the mRNA derived from that plasmid. This indicated that the band was in fact mRNA derived from the plasmids and that the mRNA was in fact from the correct genes. Fig. 3.7 represents the Northern-blot assay performed with the transcribed V(2.0)VP7-3.



**Fig. 3.7** Northern-blot assay showing the mRNA derived from pV(2.0)VP7-3. Lane (a) consists of the marker with sizes as indicated. Lanes (b - e) contain transcription reactions from p9.8-V(2.0) (b), pV(2.0)NS3-2 (c), pV(2.0)VP6-3 (d) and pV(2.0)VP7-3 (e). As  $^{32}\text{P}$ -probes against only the VP7 gene and the size marker was added, hybridization occurred only in lanes (a) and (e). The arrow indicate the position of the mRNA.

### 3.3.4 Protein-binding assays by analysis of the protein/RNA complexes on sucrose gradients

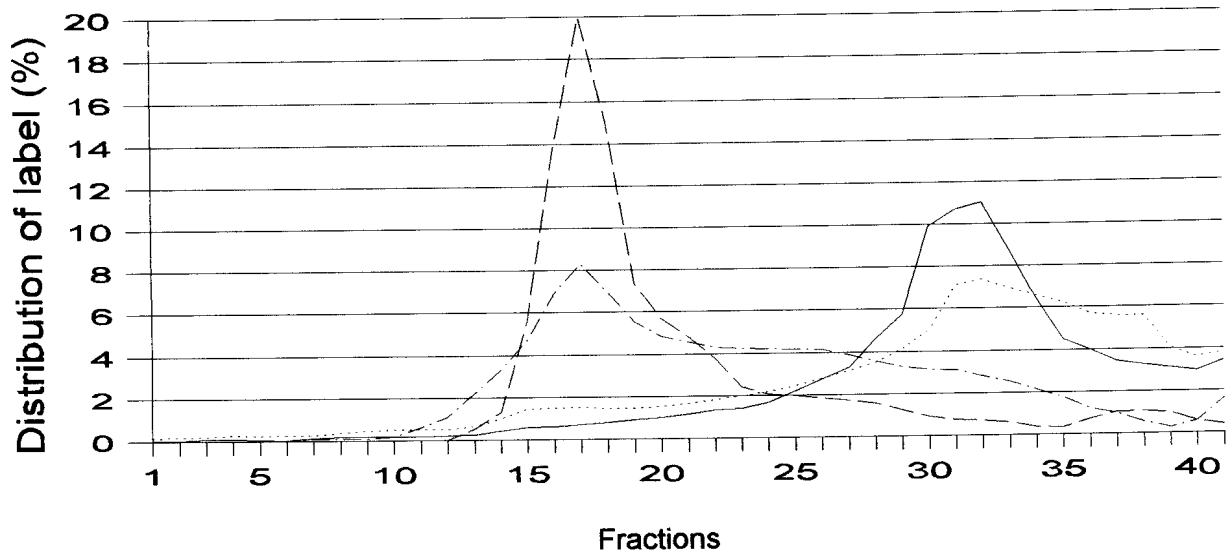
Some preliminary binding assays between the individual mRNAs and NS2 protein was carried out. First it was necessary to produce [<sup>35</sup>S]-methionine labelled NS2 protein (section 2.2.13) and [<sup>3</sup>H]-mRNA (section 3.2.7). Following *in vitro* transcription and agarose gel electrophoresis, the bands consisting of the specific [<sup>3</sup>H]-mRNA were excised from a 1 % agarose gel and purified from the gel slices with the RNaid<sup>R</sup> KIT as described in section 3.2.10. The binding assays were then carried out as described in section 3.2.11. The labelled mRNAs were mixed with the labelled proteins and the proteins were allowed to bind to the mRNAs. The fractions containing the mRNA-protein complexes were loaded on 10-40 % sucrose gradients in 0.15 M STE and centrifuged for 4.5 h at 325 000 g.

The following graphs, figures 3.8 and 3.9, show the fractions collected from the bottoms of the sucrose gradients at five drops per fraction. In each case the bottoms of the gradients are to the left and the tops to the right. Liquid scintillation counting was used to determine the amount of radioactivity in each sample. The majority of the <sup>3</sup>H was counted in the window 0-400 and the majority of the <sup>35</sup>S was counted in the window 400-700. For each fraction the background, obtained from sucrose alone, was deducted. A correction was also made to compensate for the overflow of <sup>35</sup>S in the 0-400 window. The radioactively labelled protein/mRNA in each fraction is presented as a percentage of the total amount of radioactive labelled protein/mRNA in all the fractions.

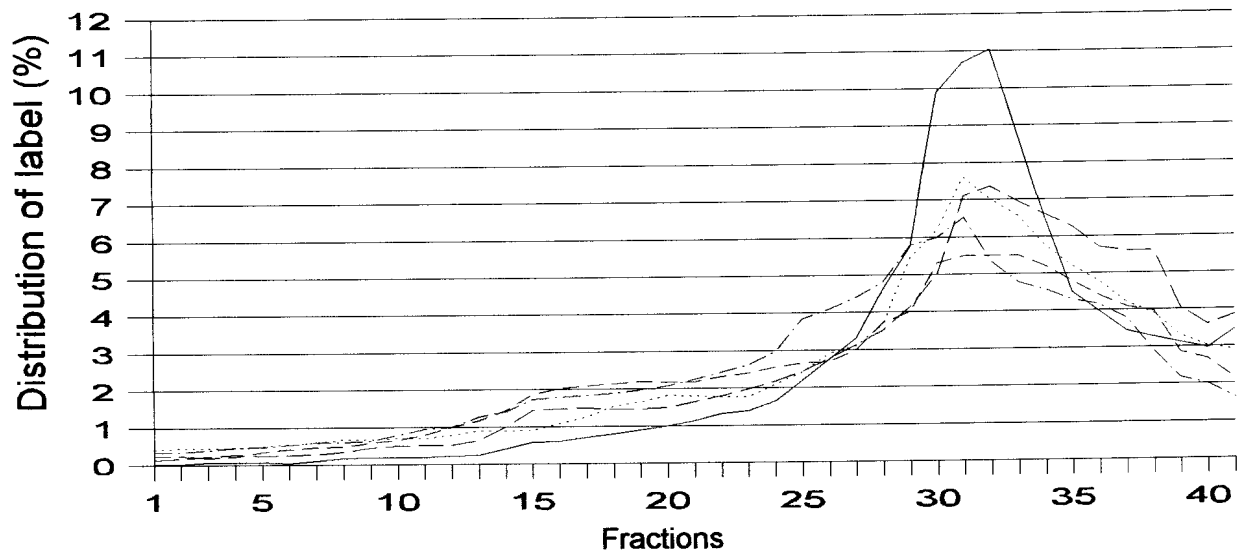
From Fig 3.8 it is evident that NS3 mRNA alone forms a distinct peak in the region of fraction 17. NS2 by it self forms a peak between fractions 30 and 34. Mixing NS2 protein with NS3 mRNA results in an increase in protein label in fractions 15 - 27 and a reduction in label in the protein peak between fractions 30 - 34. There was also an unexpected reduction in the mRNA peak and an increase in smaller mRNA fractions. This is most probably because of mRNA degradation. The same experiment was repeated for all seven mRNAs shown in Fig. 3.6.

Fig. 3.9 shows the sedimentation profiles of [<sup>35</sup>S]-methionine labelled NS2 protein mixed with various mRNAs in comparison with NS2 protein by itself. Since most of the NS2 sedimentation profiles looked the same only a few were plotted in Fig. 3.9.





**Fig. 3.8** Sucrose gradient analysis of a protein/RNA complex formed from mixing [ $^{35}\text{S}$ ]-methionine labelled NS2 with  $^3\text{H}$  labelled NS3 mRNA. The  $^{35}\text{S}$  NS2 is shown (.....) and the  $^3\text{H}$  labelled NS3 mRNA (-•-•-). Controls are unbound  $^{35}\text{S}$  NS2 (—) and unbound NS3 mRNA (- - - -).



**Fig. 3.9** Sucrose gradient analysis of a protein/RNA complex formed from mixing [ $^{35}\text{S}$ ]-methionine labelled NS2 with different  $^3\text{H}$  labelled mRNAs.  $^{35}\text{S}$  NS2 mixed with mRNA derived from pV(2.0)NS3-2 (— — —),  $^{35}\text{S}$  NS2 mixed with mRNA derived from pV(2.0)VP7-1 (.....),  $^{35}\text{S}$  NS2 mixed with mRNA derived from pV(2.0)VP7-5 (-•-•-) as well as  $^{35}\text{S}$  NS2 mixed with mRNA derived from both pV(2.0)VP7-1 and pV(2.0)VP7-3 (- - - -). Unbound  $^{35}\text{S}$  NS2 (—) was used as control.

The different results were summarised in Table 3.1 by calculating the percentage increase in [<sup>35</sup>S]-methionine labelled NS2 in fractions 15-27 and decrease in [<sup>35</sup>S]-methionine labelled NS2 in fractions 28-40 in the case of each of the different mRNAs. In each case the amount of label increase or decrease was calculated as a percentage of the amount of label observed in fractions 15-27 and 28-40 when NS2 alone was sedimented through a sucrose gradient. This represents at best a very rough (and probably still inaccurate) method to quantify the shift in protein to higher S values as the result of the addition of different mRNAs. The increase in label in fractions 15-27 could at best be considered as some indication that binding to mRNA had taken place. All mRNAs seemed to bind although the results vary from a high of 85% to a low of 7%. There was no difference with mRNA transcripts in the wrong orientation or transcripts with incomplete ends. Even when NS2 was presented with a mixture of AHSV specific mRNA with authentic ends, and nonspecific mRNA, derived from S7 cloned in the wrong transcriptional orientation, the NS2 bound. The fluctuation in the amount of NS2 increase in fractions 15-27 is less likely to be determined by the type of mRNA but much more likely due to differences in mRNA concentrations. The smaller the amount of mRNA that were present in the binding assay the smaller the increase. The binding ability of NS2 seems thus to be dependent on the mRNA concentration irrespective the source of the mRNA.

	% Increase in label in fractions 15-27	% Decrease in label in fractions 28-40
NS2 protein alone	0	0
NS2 mixed with mRNA derived from p9.8-V(2.0)	14	18
NS2 mixed with mRNA derived from pV(2.0)NS3-2	35	9
NS2 mixed with mRNA derived from pV(2.0)NS3-3	39	15
NS2 mixed with mRNA derived from pV(2.0)VP6-3	7	12
NS2 mixed with mRNA derived from pV(2.0)VP7-1	32	15
NS2 mixed with mRNA derived from pV(2.0)VP7-5	85	27
NS2 mixed with mRNA derived from both pV(2.0)VP7-1 and p9.8-V(2.0)	39	19
NS2 mixed with mRNA derived from both pV(2.0)VP7-1 and pV(2.0)VP7-3	65	27

**Table 3.1** Table showing the % increase in label in fractions 15-25 as well as the % decrease in label in fractions 28-40 for the sedimentation of [<sup>35</sup>S]-methionine labelled NS2 mixed with various mRNAs through sucrose gradients.

During the preliminary protein-binding assays many mistakes were made that should be rectified when the experiments are repeated. Each experiment should be done with a pool of mRNA of known concentration. The window that was used to count the amount of <sup>3</sup>H in each fraction was too large, a window of 0-350 would have resulted in less overflow of <sup>35</sup>S and a smaller correction would have been necessary. To prevent mRNA degradation RNase inhibitor should be added to the protein-mRNA binding reaction. As NS2 binds to both AHSV specific mRNA and nonspecific mRNA, this assay cannot be used for competition studies between mRNAs of different origin. The protein will bind to the mRNA and move towards the mRNA peak, but there is no way to determine to which mRNA it bound. It is also essential to first determine the conditions at which the mRNA rather than the protein is in excess.

### 3.4 DISCUSSION

The aim of this part of the study was to construct recombinant transcription vectors, using transcription vector V(2.0), that would provide us with individual mRNA transcripts of AHSV genes with authentic 3' and 5' ends. Some preliminary binding assays between NS2 and different mRNAs with authentic ends were also carried out. As NS2 binds to mRNA in a nonspecific way (Huismans *et al.*, 1987b; Uitenweerde *et al.*, 1995), only AHSV mRNAs with authentic ends can be used to determine if NS2 also has a preference for AHSV specific mRNAs.

In this study recombinant V(2.0) vectors containing the NS3, VP6 and VP7 genes of AHSV were produced. In order to verify whether the recombinant vectors contained full length copies of the different genes, some of the clones that contained inserts were sequenced. Recombinant vectors containing full length copies of all three genes in the right orientation for transcription were obtained as well as recombinant vectors containing full length genes in the wrong orientation of the VP6 and VP7 genes. Other gene copies were also obtained that were used in later experiments were a VP7 clone in the correct orientation that lacked two base pairs at the 5' end and a NS3 clone in the correct orientation that lacked three base pairs at the 3' end. *In vitro* transcription was performed on several of the recombinant transcription vectors, including p9.8-V(2.0) that contains the NS2, in order to obtain mRNA of the different AHSV genes. The presence of mRNA was also confirmed by Northern-blot analyses.

In the preliminary binding assays NS2 was mixed with the different mRNAs with authentic ends as well as the mRNAs containing terminal deletions and the nonspecific mRNA derived from S7 cloned in the wrong orientation. NS2 was also mixed with both specific mRNA and nonspecific mRNA. NS2 bound to all the different mRNAs that it was presented with. While NS2 does show affinity for authentic AHSV mRNAs by binding to them, it does not seem to show any preference for authentic AHSV mRNA above nonspecific mRNA. This is not too surprising as no specific binding ability for virus mRNA could thus far be shown for NS2 while there is a lot of evidence that the protein has a nonspecific binding ability (Huismans *et al.*, 1987b).

One question that is still unanswered is whether NS2 is involved in encapsidation. Because NS2 has a ssRNA binding ability but not a dsRNA binding ability it was thought that NS2 must be involved in the selection and condensation of the different ssRNA genome segments during virus encapsidation. Because very little information is available for AHSV it is important to look at analogous studies performed in other viruses.

Fujimura *et al.*, (1992) presented a model for the encapsidation of the L-A dsRNA virus of *Saccharomyces cerevisiae*. According to this model the RNA packaging domain of the Pol region of the virus's Gag-Pol fusion protein first recognises the positive strand RNA packaging signal and binds to it with its binding (polymerase) domain. The Gag domain then serves to prime capsid assembly by its homologous association with other coat protein monomers. In the closed particle negative strands are produced to form dsRNA and then again positive strands that are extruded from the particle to begin the cycle again.

Infectious bursal disease virus (IBDV) is a dsRNA virus with a genome consisting of two segments. The external surface of the virus is built up of trimeric subunits formed by a protein VP2 and the inner surface is built up of trimeric subunits formed by a protein VP3. The positively charged C terminus of VP3 might interact with the genomic dsRNA molecules. VP1, the RNA-dependent RNA polymerase, is found linked to both ends of the dsRNA genome segments. Virus like particles (VLPS) are readily formed in the absence of VP1 but it was found that VP1 is efficiently incorporated into newly formed VLPS when both VP1 and the polyprotein are co-expressed. VP3 acts as an anchor, interacting with both VP2 and VP1. The VP3-VP1 complexes are located in the inner cavity of the capsid. The interaction between VP3 and VP1 is also responsible for the incorporation of VP1 into the VLPS. These subviral complexes might bind to positive sense ssRNA before VLP formation and thus be responsible for the encapsidation of the virus genome (Lombardo *et al.*, 1999).

BTV core-like particles (CLPS) exhibited a nonspecific ability to bind nucleic acid and this ability resides on the VP3 protein. Studies with some other members of the *Reoviridae* have shown that during viral assembly positive-sense ssRNA interacts with subviral particles. In rotaviruses, RNase-sensitive ssRNA molecules have been found extending from the surface of the subviral replicative intermediates. The RNA subsequently moves into the particle and is replicated while the remaining

structural proteins assemble to form the single-shelled particle. The ability of BTV CLPs to bind external nucleic acid may have a role at an early stage in morphogenesis, during the incorporation of ssRNA into a replicative intermediate, or during assembly of VP3 onto a preformed RNA-protein complex (Loudon & Roy, 1992).

In BTV the transcription complexes lie along the 5-fold axis of the virus, at the heart of the RNA spirals, and below the pores in the VP3 layer which if slightly expanded from the resting structure would allow the exit of the RNA. These pores are lined with four arginines from each five-fold-related VP3A, of which three are strictly conserved across the orbiviruses, whereas the other is found in BTV, AHSV and EHDV. These positively charged groups may steer the RNA electrostatically, and may be important in stripping counter ions from any RNA entering or exiting the particle (Grimes *et al.*, 1998). VP4, which has capping activity, lies immediately within the VP3 layer, forming strong interactions with residues 307-328 of VP3A. Internal to VP4, and stabilised in part by interactions with the N-terminus of VP3A, lies the monomeric VP1 viral transcriptase (Gouet *et al.*, 1999). VP1 is also known to bind nucleotides, specifically to the 3' end of viral mRNA (Patton & Chen, 1999). It is probable that the enzymatic components attach to VP3 decamers, so that upon formation of the complete subcore there is a transcription complex at each of the 12 fivefold axes (Grimes *et al.*, 1998). Evidence from the cytoviruses also suggests that each genome segment may be specifically associated with a single transcriptase complex situated at one of the vertices of the intact icosahedral virus particle (Yazaki & Miura, 1980).

According to Gouet *et al.*, (1999), chemical featureless grooves on BTV VP3 form tracks for the RNA on the inside of the VP3 layer. There is evidence for specific RNA/protein interactions at two points in the icosahedral asymmetric unit between VP3 and the RNA. This paucity of specific interactions appears to facilitate the movement of RNA within the core, for example during transcription, while the counter ions lubricate the RNA/VP3 interface and the interactions between adjacent RNA layers. All the viral dsRNA segments are transcribed simultaneously at a high speed.

BTV NS2 appears to have the highest affinity for the 3' regions of the viral mRNA. Although the various ssRNA species competed for binding to NS2 during a competition assay, quite different binding affinities were observed at low molar excesses of the competitor RNAs. BTV NS2 appeared to interact preferentially with BTV S8 RNA. Rabies virus RNA competed very inefficiently

for binding to BTV NS2, while a level of interaction was detected with EHDV S8 RNA. The EHDV mRNA may have structures that not only react with BTV NS2, but may also react with its own NS2 (Theron & Nel, 1997). The mRNAs used were not mRNAs with authentic ends but runoff transcripts. The different ends of the runoff transcripts were then removed by restriction enzyme digestion before the binding assays were performed.

VP1 of rotavirus is the candidate RNA polymerase and requires the presence of VP2, the BTV VP3 analog, for replicase activity. VP1 specifically recognises the 3' end of the gene 8 mRNA. The fact that VP1 has both nonspecific and specific RNA-binding activity was previously noted for this protein and is an expected feature of RNA polymerases, since they not only have to recognize a promoter but also must move along a DNA or an RNA template during RNA synthesis. The inability of VP1 to bind to dsRNA suggests that, for the RNA polymerase to initiate transcription the 5' end of the dsRNA templates must first undergo denaturation by a helicase (Patton & Chen, 1999). Analysis of intracellular subviral particles of rotavirus led to the identification of three distinct species of replication intermediates (RIs) in infected cells: The precore RI (VP1 and VP3 the guanylyltransferase), core RI (VP1, VP2, and VP3) and VP6 RI (VP1, VP2, VP3 and VP6 the protein that forms the intermediate shell of the virus). Relatively large amounts of NSP2 co-purified with core and VP6 RIs while NSP1 and NSP3 reproducibly co-purified with precore RIs. NSP5 may also be a component of core RIs (Patton, 1995). After rotavirus infection serum IgA antibodies are predominantly directed against VP2, VP6 and NSP2. Fecal sIgA also showed major responses to VP6 and NSP2, weak reactions against VP1, VP2, NSP3 and NSP5 were also detected (Colomina *et al.*, 1998). This is further evidence for subviral particles.

Rotavirus NSP2 forms multimers and has a nonspecific ssRNA binding capacity, furthermore NSP2 interacts with VP1 the viral polymerase and is a component of the replicase complex. NSP2 is also associated with NSP5, a glycosylated phosphoprotein that is hyperphosphorylated by interaction with NSP2. In transfected cells NSP2 and NSP5 drive the formation of viroplasm-like structures in the absence of any other rotaviral protein and of rotavirus replication. Phosphorylation of NSP5 is important for the formation of viroplasms-like structures. The N-terminal region of NSP5 is likely to interact with NSP2 (Fabbretti *et al.*, 1999; James *et al.*, 1999). NSP2 multimers have an associated nonspecific NTPase activity and is also phosphorylated. NSP2 may function as a molecular motor by binding viral mRNA and catalysing its packaging through the energy generated

by its NTPase activity (Taraporewala *et al.*, 1999).

Encapsidation requires that a specific site or sites on the viral mRNA be recognised by a viral protein or proteins (Ribas *et al.*, 1994a). Rotavirus NSP3 binds to the 3' of all eleven rotavirus mRNAs and protects the whole 3' end consensus sequence from RNase T<sub>1</sub> digestion (Poncet *et al.*, 1993). NSP3 also interacts with a cellular eukaryotic translation initiation factor eIF4GI. The C-terminal half of NSP3 interacts with the N-terminal region of eIF4GI. The binding of NSP3 on eIF4GI and its specific interaction with the 3' end of viral mRNA brings the viral mRNA and the translation initiation machinery in contact, thus favouring the synthesis of viral proteins. The linking of the 3' end of mRNAs to eIF4GI is indispensable for an efficient translation initiation and strongly support the closed-loop model of mRNA translation (Piron *et al.*, 1998). NSP3 binds very strongly to eIF4GI in the absence of mRNA, this will induce the shutoff of translation of cellular mRNAs. Simultaneous interaction of NSP3 with viral mRNA and eIF4GI will allow the efficient translation of the viral mRNAs (Piron *et al.*, 1999). NSP3 recognises only those mRNAs that are derived from the same virus serogroup as the protein (Patton, 1995).

Rotavirus NSP1 has a specific affinity for all eleven rotavirus mRNAs. The target for the binding activity has been shown to reside near the 5' end of the mRNAs and may include residues located within the first 34 nucleotides of the mRNAs. NSP1 plays some beneficial role in rotavirus replication (Patton, 1995). Specific interaction between hepatitis C virus NS5B RNA polymerase and the 3' end of the viral RNA was also shown (Cheng *et al.*, 1999).

Patton (1995) suggested the following sequence for interaction of the rotavirus RNA-binding proteins with RNA during genome replication and virus assembly: First NSP3 binds to the 3' end of the virus mRNA and NSP5 binds to the 5' end of the mRNAs in the cytosol and assembles on the cytoskeleton. Precore RIs form by the addition of VP1 and VP3 and because VP3 probably binds a target that overlaps that of NSP1, the interaction of VP1 and VP3 with the NSP1/NSP3-mRNA complex would presumably competitively displace NSP1 and NSP3, respectively, from the mRNA. By displacing NSP1 and NSP3, the mRNA will no longer be anchored to the cytoskeleton and the mRNA and its associated proteins will be free to move to the viroplasm. The core RI forms as a result of the movement of the precore RI to the viroplasm. Here NSP2 and VP2 will bind to the precore RI. NSP2 may condense or organise the mRNA in preparation for packaging into cores



and RNA replication. NSP5 also interacts with NSP2 at this stage. The maturation of the core RI into VP6 RI is driven by the affinity of VP6 for VP2.

In vesicular stomatitis virus (VSV) the nucleocapsid protein N is important for RNA encapsidation. Encapsidation by the N protein initiates within the first 14 nucleotides of nascent leader RNA, which contains A residues repeated at every third base. The N protein also forms complexes with the phosphoprotein P, the P protein may impart specificity to the N protein to encapsidate the VSV genomic RNAs. The C-terminal 10 amino acids of the P protein and the C-terminal 5 amino acids of the N protein are critical for N-P interaction. The N-P complex is also essential in keeping the N protein in a replication-competent form. The N protein interacts with the growing RNA chains during replication and encapsidates both full-length plus- and minus-strand genome RNA from 5' to 3' direction. The C-terminal penultimate lysine of the N protein has a critical role in encapsidation, deletion of this single amino acid residue results in total abrogation of the encapsidation activity (Das *et al.*, 1999). Just like VSV protein N does rabies virus protein N preferentially interact with positive strand leader RNA, but unlike VSV protein N is rabies virus protein N phosphorylated while protein P is less phosphorylated. It was found that the phosphorylation of the rabies virus protein N regulates viral transcription and replication by modulating the leader RNA encapsidation (Yang *et al.*, 1999).

Assembly of replication-competent hepadnavirus nucleocapsids requires interaction of core protein, viral polymerase and a packaging signal ( $\epsilon$ ) on the pregenomic RNA. Human hepatitis B virus (HBV) core proteins can self-assemble into nucleocapsid-like particles and can encapsidate RNA nonspecifically. The C terminus of the core protein is required for pregenome encapsidation and modulates the activity of polymerase for reverse transcription of the RNA genome. The protamine-like domain located on the C-terminal of the core protein (SPRRR) is essential for pregenome encapsidation. In HBV core protein, polymerase and pregenome also interact to assemble into replication-competent nucleocapsids (Hui *et al.*, 1999).

The following model for wound tumor virus (WTV) was proposed by Anzola *et al.* in 1987 to ensure that only one of each virus RNA segment is packaged per newly synthesised virus. Each RNA segment must contain at least two operational recognition sequence domains ("sorting signals") that enables it to be recognised by the packaging proteins: one that specifies that it is a viral and

not a cellular RNA (perhaps the conserved terminal sequences), and a second that specifies that it is a particular RNA segment (perhaps the segment-specific inverted repeats). Packaging of the 12 individual WTV segments must involve 12 different and specific protein-RNA and/or RNA-RNA interactions. Sequence information required for replication and packaging of a genome segment is located within the terminal domains. Packaging of one pair of terminal structures excludes the packaging of a second copy of the same pair of terminal structures. All genome segments of a particular member of the *Reoviridae* share common 5' and 3' terminal sequences consisting of 4-8 nucleotides. In the case of the WTV genome, segment-specific sequences analogous to inverted repeats have been identified immediately adjacent to the common terminal sequences. Inspection of sequences published for the genome RNAs of other members of the *Reoviridae* indicates that segment-specific inverted repeats within the terminal sequence domains are a common feature of genomes of this virus group. This provides compelling evidence that both the conserved terminal sequences and segment-specific domains of inverted complementarity (inverted repeats) play a significant role in the sorting and assembly of segmented RNA genomes (Anzola *et al.*, 1987). The presence of inverted repeats adjacent to the conserved terminal sequences was also identified in orbivirus genome segments, including AHSV. This confirms the importance that the secondary structure of mRNA could have in events such as packaging of RNA (Van Staden *et al.*, 1991).

From our results it seems that AHSV NS2 does not only have an affinity for virus specific mRNA but also for any mRNA it is presented with. From our preliminary binding assays NS2 does not seem to show any preference for virus specific mRNA over nonspecific mRNA as is suggested to be one of its functions. This can however not be concluded from our preliminary binding assays, and can only be determined by competition studies under conditions where the mRNA is in excess of the protein and both are quantified.

NS2 is the AHSV phosphoprotein (Eaton *et al.*, 1990) and is responsible for VIB formation (Uitenweerde *et al.*, 1995). NSP5 and NSP2 of rotavirus are also both phosphorylated (Taraporewala *et al.*, 1999) and are responsible for the formation of viroplasm-like structures (James *et al.*, 1999). NS2 forms multimers (Uitenweerde *et al.*, 1995) and so does rotavirus NSP2 (Taraporewala *et al.*, 1999) and rotavirus NSP3 (Patton, 1995). NS2 contains a consensus sequence (I/L)XXM(I/L)(S/T)XXG (Van Staden *et al.*, 1991) that is also present in rotavirus NSP3 (Patton, 1995).

We suggest that NS2 is primarily involved in VIB formation. NS2 might play a role in encapsidation by binding nonspecifically to the mRNAs in the infected cell and thus presenting it near the VIBs.

From the literature it can be seen that the RNA-dependent polymerases are the only proteins with definite specificity for all ten the virus mRNAs (Patton & Chen, 1999), although not yet proven this will also most likely be the case for AHSV VP1. BTV VP3 is the main protein responsible for the organisation of the mRNA in the virion, VP3 also has specific interaction with virus mRNA (Gouet *et al.*, 1999), as all the other analogous proteins of BTV and AHSV have the same properties and functions it should be the same for VP3. There is a lot of evidence for the formation of subviral particles consisting of the polymerase and a nucleocapsid protein in *Reoviridae* (Loudon & Roy, 1992), rotavirus (Patton, 1995), IBDV (Lombardo *et al.*, 1999), VSV (Das *et al.*, 1999), HBV (Hui *et al.*, 1999) and the L-A dsRNA virus (Fujimura *et al.*, 1992).

We suggest for AHSV encapsidation that NS2 may bind nonspecifically to the mRNA in the infected cell and thus present the mRNA at the VIBs. Subviral particles consisting mainly of VP3 and VP1 then select the virus specific mRNA from the mRNA presented by NS2 and bind specifically to the virus mRNA. The main specificity thus most probably lies with VP1. VP4 and VP6 may also be part of the subviral particles that select the virus specific mRNAs or may bind to the complex after the mRNA selection. The VP3 proteins of different subviral particles then interact to form CLPs. In this way both the virus specific mRNA and the transcription complex are encapsidated simultaneously and each mRNA is associated with its own transcription complex as was suggested by Yazaki & Miura (1980). The presence of inverted repeats adjacent to the conserved terminal sequences was identified in orbivirus genome segments. This suggests that secondary structures of mRNA could play an important role in events such as packaging of RNA (Van Staden *et al.*, 1991). These inverted repeats may be important as sorting signals that distinguish between the different virus specific mRNAs. AHSV may thus follow the same model as was suggested for WTV by Anzola *et al.* (1987) in which case the packaging of one genome segment excludes the packaging of a second copy of the same genome segment. Subviral particles may also play a role in the prevention of the packaging of more than one copy of the same genome segment as the organisation of the RNA in the virus core is ordered by VP3 (Gouet *et al.*, 1999). As different subviral particles associate with the different viral specific mRNAs the proteins may fold in a unique way for each of the different mRNAs. The structural differences

between different subviral particle/mRNA complexes may prevent the association of different subviral particles containing the same genome segment. The virus might be constructed like a puzzle where only one of each piece in the correct place will complete the picture. It was reported that the virus capsid is built up from two structurally distinct copies of VP3 and that there are differences in the interactions each of these makes with the RNA that reflect specific conformational changes between the two protein subunits (Grimes *et al.*, 1998; Gouet *et al.*, 1999). The positions of VP3A and VP3B may thus be responsible for the conformational differences between the different subviral particle/mRNA complexes that make them unique.

## CHAPTER 4

### CONCLUDING REMARKS

In this study two different aspects of the ssRNA-binding nonstructural protein NS2 of AHSV were studied. Firstly the role of protein structures in ssRNA binding was examined and secondly the protein's binding ability to different mRNAs was investigated.

The first aim of this study was to determine if the difference between the ability of AHSV NS2 and BTV NS2 to bind nonspecifically to poly(U)Agarose can be ascribed to differences in the  $\alpha$ -helix rich C-terminal of NS2. In order to reach this aim a chimeric NS2 protein, consisting of the N-terminal region of AHSV NS2 and the  $\alpha$ -helix rich C-terminal of BTV was expressed in the novel BAC-TO-BAC™ protein expression system in order to produce large amounts of the protein. The binding ability of the chimeric NS2 was then compared to that of AHSV NS2 as well as that of BTV NS2 in order to determine whether the  $\alpha$ -helix rich C-terminal of the proteins are responsible for ssRNA binding. The second aim of this study was to prepare individual AHSV mRNAs with authentic 3' and 5' ends by constructing a series of recombinant vectors. Transcription vector V(2.0), utilising an internal ribozyme, was used to reach this aim.

Large amounts of chimeric NS2 was produced and its binding ability to ssRNA was compared to that of AHSV NS2 and BTV NS2. It was found that the chimeric NS2's affinity for ssRNA compares to that of AHSV NS2 and not to that of BTV NS2 from which it received its  $\alpha$ -helix rich C-terminal. We concluded that the  $\alpha$ -helix rich C-terminal is not involved in ssRNA binding but that the binding affinity rather resides on the N-terminal, and thus the first aim of this study was met.

Recombinant V(2.0) vectors containing the full length NS3, VP6 and VP7 genes of AHSV were constructed and virus specific mRNA was transcribed from these vectors as well as from p9.8-V(2.0) that contained the full length NS2 gene. Recombinant V(2.0) vectors containing the full length genes of VP6 and VP7 in the wrong orientation as well as a VP7 clone in the correct orientation that lacks two base pairs at the 5' end and a NS3 clone in the correct orientation that lacks three base pairs at the 3' end were obtained. Preliminary protein/RNA binding assays showed that AHSV NS2 has an affinity for all four different virus specific mRNAs. The protein also

bound to the VP7 gene in the wrong orientation, the mRNAs containing the deletions as well as to both mRNAs when virus specific mRNAs were mixed with nonspecific mRNAs. These studies show that NS2 binds, at least in a nonspecific manner, to all the mRNAs it was presented with. The fluctuation in the binding results also indicated that mRNA concentration plays some role in the protein/mRNA interaction.

Although there is still no undisputable evidence for the model we suggested the following: NS2 binds mRNA in the infected cells in a nonspecific manner to present it at the VIBs to subviral particles consisting of VP3 and the transcription complex to select it in a specific manner. The subviral particles bind specifically to the different virus specific mRNAs and this changes the conformation of the subviral particles in a unique way for each different genome segment. Only one each of the different subviral particle/mRNA complexes can associate with each other through their VP3 proteins to form a complete virus core consisting of only one copy of each genome segment.

One question that should still be investigated is whether the mRNA associated with the VIBs is only virus specific mRNA or a mixture between virus specific and nonspecific mRNA. As VIBs are constructed from only NS2, this will give an indication if NS2 has specificity for AHSV mRNA.

The different AHSV specific mRNAs with authentic ends that were constructed in chapter three of this study should be used in a competition assay along with nonspecific mRNA, after NS2's binding saturation thresholds for both have been determined. This will answer the question of whether AHSV NS2 has any preference for AHSV specific mRNAs over nonspecific mRNAs. The possibility of NS2 having preference for AHSV specific mRNAs is very low as no significant specific binding has thus far been shown for the protein. The studies performed by Theron & Nel (1997) where they showed that BTV NS2 shows preference for the 3' of BTV mRNAs were performed using a runoff transcript of only one of the BTV genes. The different ends of the runoff transcripts were removed by restriction enzyme digestion before the binding assays were performed.

Although there is a lot of indirect proof from BTV that AHSV VP1 is the RNA-dependent polymerase, no polymerase activity could thus far be shown for AHSV VP1, nor whether the protein has any specific mRNA affinity for AHSV specific mRNAs. It has been proposed that the

unique conserved inverted terminal repeat sequence features might play a role in sequence-specific recognition by one or more viral proteins (Van Staden *et al.*, 1991). It will thus be interesting to know whether AHSV VP1 has a preference for these repeat sequences.

Another question to which no answer has still been obtained is whether each AHSV genome segment is associated with its own transcription complex. This seems most likely to be the case.

Whether the mRNA binds to subviral particles in AHSV infected cells should also be investigated. If that is the case the specific proteins of which those particles consist must be determined. Whether the subviral particles bound to the different virus specific mRNAs have different conformational structures so that one of each is necessary for the formation of a complete virus core, must also be investigated.

**Parts of the results presented in this thesis have been presented as a poster:**

Grobler, G.C., Van Staden, V. & Huisman, H. *Structural factors that affect the binding of protein NS2 of African horsesickness virus to single stranded RNA*. The Seventeenth Congress of the South African Genetics Society, Pretoria, 27 - 30 June 2000.

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