

**Binding determinants in the interaction of the Orbivirus non-structural protein
NS2, with single-stranded RNA**

by

**Wanda Markotter
(9316361)**

**Submitted in fulfillment of the requirements for the degree
Magister Scientiae
Faculty of Natural and Agricultural Sciences
Department of Microbiology and Plant Pathology
University of Pretoria
Pretoria**

**Supervisor: Prof. L.H. Nel
Co-supervisor: Dr. J. Theron**

July 2002

ACKNOWLEDGEMENTS

I wish to express my appreciation to the following people:

Prof. L.H. Nel for his motivation, patience and support

Dr. J Theron for his interest and guidance

My parents and friends for their support

Members of the Department of Microbiology and Plant Pathology for their encouragement

The National Research Foundation for financial support

SUMMARY

Binding determinants in the interaction of the Orbivirus non-structural protein NS2, with single-stranded RNA

By

Wanda Markotter

Supervisor: Prof. L.H. Nel
Department of Microbiology and Plant Pathology
University of Pretoria

Co-supervisor: Dr J. Theron
Department of Microbiology and Plant Pathology
University of Pretoria

Morphogenesis of orbiviruses, of which bluetongue virus (BTV) is the prototype, entails a process whereby each precursor viral particle acquires a single copy of each of the ten dsRNA genome segments. The lack of free pools of dsRNA in infected cells has led to the assumption that the viral mRNA is packaged. Thus, ssRNA-binding proteins may play an important role in this process. Orbivirus mRNA bear 5' and 3' terminal hexanucleotide sequences which are common to all gene segments as well as short segment-specific inverted repeat sequences adjacent to the conserved terminal sequences. These conserved configurations provide circumstantial evidence for some functional role in encapsidation. Secondary structure analysis of BTV mRNA transcripts have predicted base-pairing at the inverted repeat sequences which results in 5' and 3' terminal overhangs as well as segment-specific stem-loop structures.

It has previously been shown that the terminal hexanucleotide sequences are not required for optimal NS2-RNA binding. In this study, the importance of the inverted repeat sequences in RNA-protein binding was investigated. Using polymerase chain reaction (PCR) methodology, three different gene constructs lacking the 5' and/or 3' inverted repeat sequences, have been constructed. The PCR products were cloned into a transcription vector under control of the T7 RNA polymerase promoter. Cytoplasmic extracts from *Spodoptera frugiperda* cells infected with a baculovirus recombinant expressing the BTV NS2 protein and *in vitro* synthesized RNA

transcripts were subjected to nitrocellulose RNA-protein binding assays. The cumulative results suggested that the inverted repeat sequences do not influence RNA-protein binding directly, but rather indirectly by altering the secondary structure of the RNA. Secondary structure motifs may thus mediate assortment of the viral mRNAs by acting as packaging signals for a specific RNA segment.

BTV NS2 has a strong affinity for BTV transcripts, but it also binds to other single-stranded RNA species. To investigate specificity in the RNA-NS2 interaction, an IAsys biosensor was used whereby the binding could be quantified. Transcription vectors containing segment 8 genes of different orbiviruses were constructed and the NS2 protein was expressed in a prokaryotic expression system as a GST fusion protein. No RNA-NS2 binding could be indicated. This approach may in future contribute to the study of RNA-protein binding interactions, if the problems as highlighted in this study, can be overcome.

Computational secondary structure prediction of all ten BTV-10 mRNA transcripts, segment 8 transcripts of different orbiviruses and different mutant transcripts was also performed. Transcripts with intact 5' and 3' inverted repeat sequences all indicated base-pairing between the 5' and 3' ends. Secondary structure analysis of the mutant transcripts lacking the inverted repeat sequences indicated alterations in the secondary structures resulting from altered base-pairing. Conserved and unique secondary structures present on segment 8 mRNAs of different orbiviruses were identified, but their functional importance awaits further mutagenesis studies.

CONTENTS	PAGE
ACKNOWLEDGEMENTS	i
SUMMARY	ii
CONTENTS	iv
LIST OF ABBREVIATIONS	ix
LIST OF FIGURES	xi
LIST OF TABLES	xv
 CHAPTER 1	
LITERATURE REVIEW	
1.1 INTRODUCTION	2
1.2 TAXONOMY OF THE ORBIVIRUS GENUS	3
1.3 ORBIVIRUS-INDUCED DISEASES	5
1.4 VIRAL STRUCTURE	5
1.4.1 The BTV virion	5
1.4.2 The viral genome	6
1.5 VIRUS PROTEINS	9
1.5.1 Structural proteins	9
1.5.2 Non-structural proteins	11
1.6 ORBIVIRUS MORPHOGENESIS	14
1.7 RNA BINDING	15
1.8 AIMS OF THIS STUDY	17
 CHAPTER 2	
THE IMPORTANCE OF THE 5' AND 3' TERMINAL INVERTED REPEAT SEQUENCES WITHIN BLUETONGUE VIRUS mRNA FOR INTERACTION WITH THE VIRUS NON-STRUCTURAL PROTEIN NS2	
2.1 INTRODUCTION	20
2.2 MATERIALS AND METHODS	21
2.2.1 Deletion mutagenesis of the NS2-encoding gene - Preparation of mutant NS2 genes	21
2.2.1.1 PCR-based deletion of the segment-specific inverted repeat sequences	21
2.2.1.2 Agarose gel electrophoresis	22

2.2.1.3 Concentration of the PCR products	22
2.2.1.4 Restriction enzyme digestion of PCR amplicons	24
2.2.1.5 Purification of restriction enzyme digested PCR fragments	24
2.2.2 Deletion mutagenesis of the NS2-encoding gene - Preparation of vector DNA	24
2.2.2.1 Preparation of competent cells and transformation	24
2.2.2.2 Small-scale plasmid DNA extraction	26
2.2.2.3 Purification of plasmid DNA	26
2.2.2.4 Restriction enzyme digestion of vector DNA	26
2.2.2.5 Purification of restriction enzyme digested DNA fragments	27
2.2.3 Cloning of PCR fragments into pBS	27
2.2.3.1 Ligation	27
2.2.3.2 Preparation of competent cells and transformation	27
2.2.4 Selection of recombinants	28
2.2.4.1 Plasmid DNA extractions	28
2.2.4.2 Restriction enzyme digestions	28
2.2.4.3 Nucleic acid sequencing of mutant NS2 genes	29
2.2.4.3.1 Preparation of plasmid DNA	29
2.2.4.3.2 Sequencing reaction	29
2.2.4.3.3 Purification of extension products	30
2.2.4.4.4 Gel electrophoresis of purified extension products	30
2.2.5 Preparation of radioactively labelled RNA transcripts	30
2.2.5.1 Linearization of recombinant plasmid DNA	30
2.2.5.2 Purification of linearized DNA fragments	30
2.2.5.3 <i>In vitro</i> transcription	31
2.2.5.4 Precipitation of the single-stranded RNA	31
2.2.6 Expression of the BTV NS2 protein	31
2.2.6.1 Cells and viruses	31
2.2.6.2 Virus titration	32
2.2.6.3 Preparation of cell extracts	32
2.2.6.4 SDS-polyacrylamide gel electrophoresis	33
2.2.6.5 Western blot analysis	33

2.2.7 Nitrocellulose RNA-protein binding assay	34
2.3 RESULTS	36
2.3.1 Construction of mutant copies of the NS2-encoding gene	36
2.3.2 <i>In vitro</i> transcription	41
2.3.3 Expression of the BTV-NS2 protein in the baculovirus expression system	41
2.3.4 Nitrocellulose RNA-protein binding assay	43
2.4 DISCUSSION	46

CHAPTER 3

COMPARATIVE BINDING ANALYSIS OF BTV, EHDV AND AHSV SEGMENT 8 RNA TO THE BTV NS2 PROTEIN USING AN IA_{sys} BIOSENSOR

3.1 INTRODUCTION	49
3.2 MATERIALS AND METHODS	50
3.2.1 Construction of recombinant transcription vectors containing different orbivirus segment 8 genes	50
3.2.1.1 Large-scale plasmid DNA isolation	50
3.2.1.2 Restriction enzyme digestions	51
3.2.1.3 Purification of insert and vector DNA	51
3.2.1.4 Dephosphorylation of vector DNA	51
3.2.1.5 Ligation	51
3.2.1.6 Preparation of competent cells and transformation	52
3.2.1.7 Small-scale plasmid DNA isolation	53
3.2.1.8 Restriction enzyme digestion	53
3.2.1.9 Preparation of recombinant plasmids	53
3.2.2 Expression of the BTV NS2-GST fusion protein in <i>Escherichia coli</i>	53
3.2.2.1 Small-scale expression of the fusion protein	54
3.2.2.2 Large-scale expression of the fusion protein	54
3.2.2.3 Lysis of the bacterial cells	54
3.2.2.4 SDS-polyacrylamide gel electrophoresis	55
3.2.2.5 Western Blot analysis	55

3.2.3 Preparation of <i>in vitro</i> RNA transcripts	55
3.2.3.1 Linearization of plasmid DNA	55
3.2.3.2 Purification of linearized plasmid DNA	55
3.2.3.3 <i>In vitro</i> transcription	55
3.2.4 RNA-protein binding analysis using a biosensor	56
3.3 RESULTS	57
3.3.1 Cloning of full-length AHSV and EHDV NS2-encoding genes into pBS	57
3.3.2 Expression of the BTV NS2 protein in a prokaryotic expression system	61
3.3.3 RNA-protein binding experiments using the IAsys biosensor	63
3.4 DISCUSSION	67

CHAPTER 4

SECONDARY STRUCTURE ANALYSIS OF THE mRNA SEGMENTS OF DIFFERENT ORBIVIRUSES AND IDENTIFICATION OF STRUCTURAL DETERMINANTS THAT MAY BE INVOLVED IN RNA-PROTEIN BINDING

4.1 INTRODUCTION	71
4.2 MATERIALS AND METHODS	73
4.2.1 GenBank accession numbers	73
4.2.2 Computational analysis of RNA secondary structures	74
4.2.3 Detection of conserved RNA structural elements in the segment 8 RNAs of different orbiviruses	75
4.3 RESULTS	77
4.3.1 Secondary structure analysis of the full-length mRNA segments of BTV-10	77
4.3.2 Secondary structure predictions of the mutant transcripts lacking inverted repeat sequences	85
4.3.3 Secondary structure analysis of different orbivirus segment 8 mRNA transcripts and identification of conserved secondary structure motifs	91
4.3.4 Identification of unique secondary structures on BTV-10 segment 8 RNA	100

4.4 DISCUSSION	102
CHAPTER 5	
CONCLUDING REMARKS	105
REFERENCES	108
COMMUNICATIONS	123



LIST OF ABBREVIATIONS

Å	Angstrom
AcMNPV	<i>Autographa californica</i> nuclear polyhedrosis virus
AHA	aminohexanoic acid
AHSV	African horse sickness virus
arc seconds	1/60 th arc minute = 1/360 th degree of angle
ATP	adenosine-5'-triphosphate
bp	base pair
BHK	baby hamster kidney cells
BTV	bluetongue virus
CMB	calcium manganese based
CMD	carboxy methyl dextran
cpm	counts per million
CTP	cytosine – 5'-triphosphate
dd H ₂ O	double distilled water
°C	degrees Celsius
DEPC	Diethylpyrocarbonate
DNA	deoxyribonucleic acid
ds	double stranded
DTT	Dithiothreitol
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
EDTA	ethylenediaminetetra-acetic acid
EHDV	epizootic haemorrhagic disease virus
ER	endoplasmic reticulum
FCS	fetal calf serum
FMD	foot and mouth disease
GSH	Glutathione
GST	glutathione S-transferase
GTP	guanosine-5'-triphosphate
h	Hour
IPTG	isopropyl β-D-thiogalactosidase
KASV	Kasba virus
kb	kilobase pairs
KDa/K	Kilodalton
LB	Luria-Bertani
M	Molar

mA	milli-ampere
μCi	Microcurie
μg	Microgram
μl	Microliter
MOI	multiplicity of infection
Mr	molecular weight
m/v	mass per volume
ng	Nanogram
NHS	N-hydroxysuccinimide
nm	Nanometer
NS	non-structural
NTP	nucleotide triphosphate
OD	optical density
PCR	polymerase chain reaction
PEG	polyethylene glycol
PBS	Phosphate buffered saline
PSB	protein solvent buffer
pfu	plaque forming units
RGD	arginine glycine aspartate
RNA	ribonucleic acid
rpm	revolutions per minute
SA	South Africa
SDS	sodium dodecyl sulphate
PAGE	polyacrylamide gel electrophoresis
PSB	phosphate-buffered saline
s	Second
Sf	<i>Spodoptera frugiperda</i>
ss	single stranded
U	Units
UTP	uridine-5'-triphosphate
UV	Ultraviolet
V	Volts
VIB	virus inclusion bodies
WTV	wound tumor virus
TEMED	N,N,N',N'-tetramethylethylenediamine
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

LIST OF FIGURES

FIGURE NUMBER		PAGE
Figure 2.1	Schematic representation of the primers and annealing sites used during the construction of different NS2 genes lacking inverted repeat sequences.	23
Figure 2.2	Plasmid map of the pBS vector (Stratagene) used during this study as a cloning and transcription vector.	25
Figure 2.3	Diagram of the experimental procedures used in a nitrocellulose-binding assay to investigate the importance of the inverted repeat sequences in RNA-protein binding.	35
Figure 2.4	Agarose gel electrophoresis of the amplicons obtained using the primers as described in Fig. 2.1.	36
Figure 2.5	Agarose gel electrophoresis of plasmid DNA extracted from putative recombinant transformants.	37
Figure 2.6	Agarose gel electrophoretic analysis of the recombinant transcription vector after <i>Bam</i> HI restriction enzyme digestion.	38
Figure 2.7	Plasmid map indicating the possible orientations of the cloned insert DNA in the pBS transcription vector.	39
Figure 2.8	Agarose gel electrophoretic analysis of the recombinant transcription vector constructs indicating the orientation of the cloned inserts.	40
Figure 2.9	SDS-PAGE analysis of cellular extracts prepared from recombinant baculovirus-infected cells expressing BTV NS2 and mock-infected <i>Sf</i> cells.	42
Figure 2.10	Western blot analysis verifying the authenticity of the baculovirus-expressed BTV NS2 proteins.	43

- Figure 2.11 Analysis of the ssRNA-binding ability of baculovirus-expressed BTV NS2 to labelled transcripts using a nitrocellulose-binding assay. 45
- Figure 3.1 Agarose gel electrophoretic analysis of the putative recombinant pBS transcription vectors following *Bam* HI restriction enzyme digestion. 58
- Figure 3.2 Plasmid map indicating the possible orientation of the cloned EHDV segment 8 DNA in the pBS transcription vector. 59
- Figure 3.3 Plasmid map indicating the possible orientation of the cloned AHSV segment 8 DNA in the pBS transcription vector. 60
- Figure 3.4 (A) Agarose gel electrophoretic analysis of the EHDV segment 8 recombinant plasmid after *Eco* RI restriction enzyme digestion to verify the orientation of the cloned insert DNA. 61
- Figure 3.4 (B) Agarose gel electrophoretic analysis of the AHSV segment 8 recombinant plasmid after *Hind* III restriction enzyme digestion to verify the orientation of the cloned insert DNA. 61
- Figure 3.5 SDS-PAGE analysis of *E.coli* extracts indicating expression of the BTV NS2 protein. 62
- Figure 3.6 Western Blot analysis indicating prokaryotic expression of the BTV GST-NS2 fusion protein. 63
- Figure 3.7 Graphical presentation of the different stages involved in preparing the CMD cuvette for use in the IAsys biosensor to study RNA-protein binding. 64
- Figure 4.1 Diagrammatic representation of the comparison of different secondary structures using the programs ALIDOT and PFRALI. 76

- Figure 4.2** Magnification of the 5' and 3' ends of BTV-10 mRNA segments 1- 78
5 indicating base-pairing at the inverted repeat sequences and
the stem-loop structure present at the 5' or 3' ends of all ten
mRNA transcripts.
- Figure 4.3** Magnification of the 5' and 3' ends of BTV-10 mRNA segments 6- 79
10, indicating the base-pairing at the inverted repeat sequences
and the stem-loop structure present at the 5' or 3' ends of all ten
mRNA transcripts.
- Figure 4.4** Secondary structure predictions of full-length RNA segments 1 80
and 2 of BTV-10 indicating base-pairing at the 5' and 3' inverted
repeat sequences.
- Figure 4.5** Secondary structure predictions of full-length RNA segments 3 81
and 4 of BTV-10 indicating base-pairing at the 5' and 3' inverted
repeat sequences.
- Figure 4.6** Secondary structure predictions of full-length RNA segments 5 82
and 6 of BTV-10 indicating base-pairing at the 5' and 3' inverted
repeat sequences.
- Figure 4.7** Secondary structure predictions of full-length RNA segments 7 83
and 8 of BTV-10 indicating base-pairing at the 5' and 3' inverted
repeat sequences.
- Figure 4.8** Secondary structure predictions of full-length RNA segments 9 84
and 10 of BTV-10 indicating base-pairing at the 5' and 3' inverted
repeat sequences.
- Figure 4.9** Magnification of the 5' and 3' terminal secondary structures of 86
mutant transcripts lacking inverted repeat sequences.
- Figure 4.10** Secondary structure analysis of the full-length BTV-10 segment 8 87
RNA transcript.

- Figure 4.11 RNA secondary structure prediction of the mutant transcript 88
i5'3'BTVNS2 from which both the 5' and 3' inverted repeat
sequences were removed.
- Figure 4.12 RNA secondary structure prediction of the mutant transcript 89
i5'BTVNS2 from which the 5' inverted repeat sequence was
removed.
- Figure 4.13 RNA secondary structure prediction of the mutant transcript 90
i3'BTVNS2 from which the 3' inverted repeat sequence was
removed.
- Figure 4.14 Secondary structure analysis of the full-length EHDV segment 8 92
RNA indicating base-pairing at the 5' and 3' inverted repeat
sequence.
- Figure 4.15 Secondary structure analysis of the full-length AHSV segment 8 93
RNA indicating base-pairing at the 5' and 3' inverted repeat
sequence.
- Figure 4.16 Secondary structure analysis of the full-length KASV segment 8 94
RNA indicating base-pairing at the 5' and 3' inverted repeat
sequence.
- Figure 4.17 Multiple sequence alignment of different orbivirus segment 8 96
RNAs indicating conserved sequences.
- Figure 4.18 Unique secondary structures present on BTV-10 segment 8 RNA. 101

LIST OF TABLES

TABLE NUMBER		PAGE
Table 1.1	Taxonomic structure of the family <i>Reoviridae</i>	4
Table 1.2	Sequence arrangements on the BTV-10 genome	8
Table 1.3	Function of bluetongue virus proteins	13
Table 2.1	Sequences of the PCR primers used to construct mutant NS2 genes lacking inverted repeat sequences	21
Table 2.2	Scintillation counts (cpm) of the labelled transcripts after <i>in vitro</i> transcription reactions and of nitrocellulose membranes after binding experiments at different salt concentrations	44
Table 3.1	Details of the events using the IAsys biosensor to study RNA-protein binding	65
Table 4.1	GenBank accession numbers of sequences used in the secondary structure analysis	74
Table 4.2	Predicted consensus secondary structures for the different orbivirus segment 8 RNAs (AHSV, EHDV, BTV and KASV)	97

CHAPTER 1: LITERATURE REVIEW

1.1 INTRODUCTION

Viruses have probably been present on earth since the origin of life. Before viruses were identified as a distinct group of infectious organisms, people were already aware of their effects. The first written record of a virus infection was found in an Egyptian hieroglyph dating back to 1400 BC, which depicts a temple priest showing typical signs of paralytic poliomyelitis. Lesions found on the face of Pharaoh Ramses V, who died in 1196 BC, provides circumstantial evidence that he may have succumbed to smallpox (Reviewed: Fraenkel-Conrat *et al.*, 1994). By the eighteenth century progress had already been made in preventing viral disease. Lady Montague, wife of the English Ambassador to Turkey, noticed that Turkish women inoculated their children against smallpox by placing smallpox pustules either under a fingernail or on the skin. The children acquired immunity against the disease and displayed only mild disease symptoms. She subsequently introduced this vaccination procedure in London during 1721 (Reviewed: Fraenkel-Conrat *et al.*, 1994). In 1796, Edward Jenner successfully began inoculating humans with material obtained from cowpox-infected material in order to protect them against human smallpox (Lustig and Levine, 1992).

However, it was only until late in the 19th century that observations were made that indicated the existence of a disease-causing agent smaller than bacteria (Reviewed: Lustig and Levine, 1992). In 1884, Charles Chamberland developed a porcelain bacterial filter and based on the different filterability properties of bacteria and viruses, a distinction could be made between them. Dimitri Ivanovski's published studies in 1892, showed that leaf extracts obtained from infected tobacco plants, and that had been filtered to remove bacteria, still induced disease in healthy plants. In 1898, Marthinus Beijerinck described the causative agent of tobacco mosaic disease as an entity different from bacteria. He referred to this new organism as *contagium vivum fluidum* (soluble living germ). At the same time, Friedrich Loeffler and Paul Frosch found that a virus was responsible for foot-and-mouth disease in cattle. Walter Reed later showed that yellow fever in humans was due to a filterable virus transmitted by mosquitoes. By the beginning of the 20th century it was established that viruses are different from bacteria and could cause diseases in humans, plants and animals. Viruses are now recognized as a unique group of infectious agents that have a simple acellular organization, consisting only of one type of nucleic acid, DNA or RNA, enclosed by a protein capsid. Since viruses are unable to independently perform metabolic processes, they are

considered to be obligate intracellular parasites. They require the host cell's machinery to synthesize their macromolecular components with progeny viruses ultimately being released from the infected host cells.

Today, many viruses of medical and agricultural importance have been identified. Members of the orbivirus genus in the family *Reoviridae* are important causative agents of livestock diseases. The prototype virus of this genus is bluetongue virus (BTV), while epizootic haemorrhagic disease virus (EHDV) and African horse sickness virus (AHSV) are also important members (Murphy *et al.*, 1971). An outbreak of bluetongue disease was probably first described by Hutcheon in 1881 (Hutcheon, 1902). Theiler suggested in 1906 that the causative agent is a virus (Howell, 1963), but it was not until 1940 that the virus could be propagated in embryonated eggs for the first time (Mason *et al.*, 1940). Verwoerd (1969) was the first to purify the virus and he demonstrated that BTV contained dsRNA by using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Since the disease was first recognized in South Africa, it would therefore appear as if it originated on the African continent and for many decades it was believed to be confined to the African continent. The first outbreak outside of Africa occurred in Cyprus (Gambles, 1949) and the disease has since been reported in many different parts of the world including North and South America, Australia and south-east Asia. BTV can replicate in vertebrate species and arthropods, while biting midges (*Culicoides* species) transmit BTV to ruminants and play a crucial role in the spread of the virus from BTV-endemic to non-BTV regions. Currently, 24 different BTV serotypes are recognized on the basis of their different reactions in serum neutralization tests (Howell 1960, 1970; Howell and Verwoerd 1971; Gorman, 1983).

1.2 TAXONOMY OF THE ORBIVIRUS GENUS

Viruses with segmented dsRNA genomes are widespread in nature and infect hosts ranging from bacteria and fungi to species throughout the plant and animal kingdoms (Reviewed: Murphy *et al.*, 1995). They belong to the families *Reoviridae*, *Birnaviridae*, *Cystoviridae*, *Totiviridae* and *Partitiviridae* (Murphy *et al.*, 1995). *Reoviridae* is the largest of these families and the best studied. Members of this virus family do not have a lipid envelope. The viral particles are large and complex with icosahedral symmetry. Their genomes consist of 10-12 dsRNA segments (except *Totiviridae*) and each viral segment encodes at least one polypeptide. In 1999, the International Committee of Taxonomy of

Viruses reported that the orbiviruses constitute one of nine distinct genera in the family *Reoviridae* (Van Regenmortel *et al.*, 2000) (Table 1.1).

Table 1.1: Taxonomic structure of the family *Reoviridae* (Van Regenmortel *et al.*, 2000)

Genus	Prototype virus	Primary host
Orthoreovirus	Reovirus 3	Mammals
Orbivirus	Bluetongue virus 1	Mammals
Rotavirus	Simian rotavirus SA11	Mammals
Coltivirus	Colorado tick fever virus	Mammals
Aquareovirus	Golden shiner virus	Fish
Cypovirus	Bombyx mori cypovirus 1	Insects
Fijivirus	Fiji disease virus	Plants
Phytoreovirus	Wound tumour virus	Plants
Oryzavirus	Rice ragged stunt virus	Plants

The orbiviruses comprise a group of arthropod-borne viruses which have similar morphological and physicochemical properties (Borden *et al.*, 1971). “*Orbis*” in Latin means ring or circle and the name of this group of viruses therefore reflects the large ring-shaped capsomers seen on the surface of the virus particles under the electron microscope following negative staining. In addition to this feature, their loss of infectivity in mild acid conditions distinguish them from other members of the *Reoviridae* family. Although orbiviruses are resistant to lipid solvents and detergents, e.g. diethyl ether, deoxycholate and chloroform (Howell, 1963; Studdert *et al.*, 1966; Bowne and Ritchie, 1970), sodium dodecyl sulphate disrupts the viral particle and destroys virus-infectivity.

Orbiviruses are differentiated into 14 serological groups and a few unassigned viruses also exist (Van Regenmortel *et al.*, 2000). Whereas viruses in each serogroup share antigens detectable in complement fixation, immunodiffusion and fluorescent antibody tests, serotypes within each serogroup are distinguished by specific reactions in serum neutralization tests (Borden *et al.*, 1971; Gorman *et al.*, 1983). Genomic reassortment has been reported to occur between some viruses within a serogroup or antigenic

complex, but not yet between members representing different serogroups (Kahlon *et al.*, 1983; Gorman *et al.*, 1983; Roy *et al.*, 1982; Samal *et al.*, 1987).

1.3 ORBIVIRUS-INDUCED DISEASES

BTV causes bluetongue disease in both wild and domestic ruminants. BTV has traditionally been regarded as a disease of sheep, but it has since become evident that its natural host range is extremely wide and it may infect sheep, cattle, goats, deer and African antelope (Hoff and Hoff, 1976). BTV causes a degenerative, sometimes-fatal disease in sheep (Neitz, 1948) and outbreaks frequently occur when new breeds of sheep are introduced into areas where BTV is endemic. Essentially the same applies for cattle and goats. The incubation period of BTV is two to seven days and the first symptom in sheep is depression followed by the rolling movement of the tongue, licking of lips, nasal discharge and salivation. The lips and tongue swell and muscle weakness and stiffness are observed (Erasmus, 1975). Other members of the orbivirus genus may also cause disease in native and domestic animals and even humans. For example, EHDV infects deer and antelope and AHSV infects horses, mules, donkeys and zebra, whilst members of the Colorado tick fever serogroups, Kemerovo serogroup, Changunda serogroup and Corriparta serogroup can infect humans.

1.4 VIRAL STRUCTURE

1.4.1 The BTV virion

The virus is non-enveloped (Verwoerd, 1969) and three morphologically distinct BTV particles can be observed: virions, cores and subcores. The negatively-stained BTV virion is about 80 nm (Verwoerd and Huisman, 1972; Martin and Zweerink, 1972) and has a fuzzy appearance because of the diffuse outer capsid. The outer capsid consists of 180 copies of VP2 protein arranged as triskellion structures and 360 copies of VP5 protein arranged as 120 trimers (Mertens *et al.*, 1984). Both VP2 and VP5 are attached to VP7 to form part of the core particle. *In vivo*, virions are uncoated to release the core particles immediately after infection. This occurs through the removal of the outer capsid protein layer (VP2 and VP5). Later in the infection cycle core particles are uncoated, by removal of VP7, forming the subcore particles. The core particles have a diameter of about 73 nm (Mertens *et al.*, 1987). A combination of three-dimensional cryo-electron microscopic analysis of the core at 25-Å resolution and X-ray crystallographic structure analysis at 3.5-Å resolution has revealed the complexity of the core arrangements. The

core is made up of 13 icosahedrally independent copies of the VP7 molecule in the form of four trimers (P, Q, R and S) in general positions (icosahedral three-fold axis) and one (T) located with its three-fold axis aligned with the icosahedral three-fold axis (Grimes *et al.*, 1997; Grimes *et al.*, 1998; Grimes *et al.*, 1995). A total of 780 copies of VP7 arranged as 260 trimers form the core surface. The three-fold axis of these trimers is perpendicular to the core surface and the broad flat base of each trimer contacts the inner subcore. The subcore (thin inner shell of the core) has a hexagonal shape and a diameter of 59 nm. They are composed of 120 copies of VP3 arranged with T=2 symmetry and enclose the three minor proteins (VP1, VP4 and VP6) as well as the ten segments of the genome. The subcore is composed of VP3 molecules and carries the information that defines the size of the virus (Grimes *et al.*, 1998). The subcore possesses small pores (Grimes *et al.*, 1998) at the icosahedral three-fold axis and larger openings at the five-fold axis. The small pores can be enlarged by structural rearrangements of amino acid side chains and this may therefore in theory serve as channels whereby viral segments can be packaged. Even so, this scenario appears to be unlikely since the VP7 trimers block these pores. The larger pores at the five-fold axis are too small for the RNA to pass through but activation of the cores for transcription leads to conformational changes that may enlarge these openings, allowing the RNA to pass through.

The BTV core particle contains ten dsRNA segments that are separately and repeatedly transcribed within the core. Individual dsRNA must be transported through the active sites of the transcriptase enzyme and must also be ready for the next round of transcription. Therefore, there must be a high level of organization within the core to avoid tangling and steric interference during movement (Gouet *et al.*, 1999). The dsRNA genome is organized in partially ordered concentric layers within the core (Gouet *et al.*, 1999) and each segment may exist as a tightly wound spiral around one RNA-dependant RNA polymerase-capping complex (Gouet *et al.*, 1999).

1.4.2 The viral genome

The BTV genome consists of a segmented double-stranded RNA genome of ten segments (Gorman *et al.*, 1983; Verwoerd *et al.*, 1970) that is packaged in equimolar ratios, i.e. one of each segment per virus particle. The total molecular weight of the BTV-10 genome is 11.8×10^6 Da (19218 bp) (Verwoerd *et al.*, 1970). The segments are

numbered one to ten in order of their size which ranges from 0.5 (822 bp) to 2.8×10^6 Da (3954 bp) (Verwoerd *et al.*, 1979). The genome encodes seven structural proteins (VP1 to VP7) and four non-structural proteins (NS1, NS2, NS3 and NS3A). Except for segment 10, each viral RNA segment encodes one of these proteins.

The genome of BTV-10 has been completely sequenced and sequencing data indicated the presence of 5' and 3' terminally conserved hexanucleotide sequences on all ten RNA segments (Rao *et al.*, 1983). At the 3' ends, the sequence 3' CAUUCA was found in all the positive strands, while 3'CAAUUU was found on all the negative strands (Mertens and Sangar, 1985). Another shared feature is the presence of inverted repeat sequences, adjacent to these conserved termini, differing in sequence and length for each segment (Cowley *et al.*, 1992). These inverted repeat sequences are 5-14 nucleotides in length and based on secondary structure predictions of the BTV mRNA segments, base-pairing at the inverted repeats may occur (Cowley *et al.*, 1992). Inverted repeat sequences have also been found in other multi-segmented RNA genome viruses, e.g. Ibaraki virus (Mertens and Sangar, 1985), EHDV (Nel *et al.*, 1990), WTV (Anzola *et al.*, 1987), reovirus type 3 (Antczak *et al.*, 1982) and influenza virus (Stoeckle *et al.*, 1987). This conserved configuration may thus serve some purpose during virus replication.

Table 1.2: Sequence arrangements on the BTV –10 genome

RNA	Protein	Length	5' end sequence	3' end sequence
L1	VP1	3954 bp	GUUAAA AUGCAAUG	UGAGAGCACGCGCGAGCACGCG CCGCAUUACACUUAC
L2	VP2	2926 bp	GUUAAAAGAGUGUUCUAC CAUG	UAGGUCCUGUGACAUGGACCGG UAGCCUCUUACACUUAC
L3	VP3	2772 bp	GUUAAAUUUCCGUAGCCA UG	UAGAUGUGCGACCGAUCUAUGC ACUUGGUAGCGGCAGCGGAAAC ACACUUAC
M4	VP4	2011 bp	GUUAAAACAUGCCUGAGC CACACGCAUG	UAAUGCGUGACUGCUAGGUGAG GGGGGCAUGUACAACUUAC
M5	VP5	1769 bp	GUUAAAAGuGUUCUCCUA CUCGCAGAAGAUG	UGAACGCAGCGGGGGAGGACCU UCCACUUAC
M6	NS1	1638 bp	GUUAAAAGUUCUcuaCU AGUUGGCAACCACCAAACA UG	UAGUUACUGAUUUUUAGUUUUU UAUUCUUCUUUUCAUUUCUAUU UUCUCUUAGCACUCUACUuAGAA CUUUUCAACUUAC
S7	VP7	1156 bp	GUUAAA AUUCUAuAGAGAU G	UAGUCCACUUUGCACGGGUGUG GGUUACAUAUGCGGUGUGUGUC GGUUGUGGGAAUAUGUAACCC AUUUAAACGUCUCUUAGAUUACA CUUAC
S8	NS2	1124 bp	GUUAAAAAUCCUUGAGUC AUG	UGACCGCAUGAUUGGGGGGGGA UUUUACACUUAC
S9	VP6	1064 bp	GUUAAAAAUUCGCaUAUGU CAGCU	UAAAGGGUCCAGGGUACCUUCU UGACGUAggGCGAUUUUCACACUU AC
S10	NS3	822 bp	GUUAAAAGUGUCGCGC CAUG	GGGUGCGCGAUACUACACACUU AC

Conserved hexanucleotide sequences (Rao *et al.*, 1983; Mertens and Sangar, 1985) are indicated in red and inverted repeat sequences (Cowley *et al.*, 1992) are indicated in italic. Lower case letters indicate mismatches in the inverted repeat sequences.

1.5 VIRUS PROTEINS

Eleven virus-specified polypeptides have been detected in BTV-infected cells (Gorman *et al.*, 1981; Huismans, 1979; Mertens *et al.*, 1984). These consist of seven structural proteins (VP1 – VP7) and four non-structural proteins (NS1, NS2, NS3 and NS3A).

1.5.1 Structural proteins

There are four major core proteins (VP2, VP3, VP5 and VP7) and three minor core proteins (VP1, VP4 and VP6).

VP2

VP2 is encoded by RNA segment 2. This protein is the major component of the outer capsid of BTV and is the main serotype-specific antigen, eliciting neutralizing antibodies (Huismans and Erasmus, 1981; Kahlon *et al.*, 1983). Due to the ability of purified BTV VP2 to elicit neutralizing antibodies in sheep, it may be a suitable candidate for a subunit vaccine (Huismans *et al.*, 1987b). BTV VP2 exhibits haemagglutinin activity (Hassan and Roy, 1999) and there is also some evidence indicating that the protein is involved in cellular attachment (Verwoerd *et al.*, 1972) and virus entry into the host cell (Hassan and Roy, 1999). Virus particles of which VP2 has been removed, is less infectious and no longer capable of binding to host cells.

VP3

VP3 is encoded by RNA segment 3 and is the major structural protein of the BTV inner shell where it forms the protein-scaffold on which the capsomeres and minor structural proteins are arranged (Huismans and Erasmus, 1981). The protein contains group-specific antigenic determinants (Huismans and Erasmus, 1981). Core-like particles synthesized from the co-expression of BTV VP3 and VP7 by baculovirus recombinants have been shown to bind ssRNA non-specifically as well as DNA (Loudon and Roy, 1992). The binding activity was subsequently shown to reside on VP3. The smallest virus particle containing RNA is VP3 subcores. Therefore, VP3 seems to play a fundamental role in the early stages of BTV core formation (Grimes *et al.*, 1998; Loudon and Roy, 1991).

VP5

VP5 is encoded by RNA segment 5. Very little is known about VP5 other than it forms part of the outer capsid and can play a contributory role in the induction of neutralizing antibodies during BTV infection (Mertens *et al.*, 1989). It has been reported that when sheep were vaccinated with a mixture of VP2 and VP5, it provides a higher neutralizing antibody response than sheep vaccinated with VP2 alone. Thus, VP5 may enhance the immune response indirectly by interacting with VP2 thereby affecting the conformation of VP2 and consequently its serological properties (Roy *et al.*, 1990b). Although VP5 binds to the cell surface, it does not appear to be responsible for internalization of the virus. The protein, however, plays a role in membrane destabilization once the virus core is released into the cytoplasm (Hassan *et al.*, 2001).

VP7

VP7 is encoded by RNA segment 7. The VP7 protein is a major structural component of BTV cores and comprises 36% of the total amount of core proteins (Huismans *et al.*, 1987b; Hyatt and Eaton, 1988). Not only does VP7 contain group-specific antigenic determinants (Huismans and Erasmus, 1981), but it also has an arginine-glycine-aspartate (RGD) tripeptide motif, one of the ligand sites recognized by integrin host proteins. This domain is accessible on the surface of the protein and is involved in binding of the BTV core to *Culicoides* cells (Xu *et al.*, 1997; Tan *et al.*, 2001). This motif has also been shown to be essential for virus entry in other viruses (Tan *et al.*, 2001), amongst other rotaviruses, adenovirus, papillomavirus and members of the *Picornaviridae* family such as FMDV.

VP1

The largest RNA segment, segment 1, encodes the minor core protein, VP1, that is present in a very low molar ratio in the core of the virions (Huismans and van Dijk, 1990). The protein is the RNA-dependant RNA polymerase (Urakawa *et al.*, 1989) and functions as the viral transcriptase. Its amino acid sequence displays high levels of similarity with other DNA and RNA virus polymerases and also with eukaryotic polymerase subunits (Roy, 1992). Nucleic acid hybridization studies have indicated that this gene is highly conserved amongst the different virus serotypes (Fukusho *et al.*, 1989).

VP4

VP4 is encoded by RNA segment 4 and is a minor protein component of the inner core. Results obtained by expressing VP4 in the baculovirus system has indicated that it binds guanosine-5'-triphosphate (GTP) and is thus proposed to be the candidate guanylyl transferase RNA capping enzyme of the virus (Le Blois *et al.*, 1992; Ramadevi *et al.*, 1998). The protein possesses nucleoside triphosphate phosphohydrolase activity (NTPase), which is important for transcription, RNA processing, translation (Ramadevi and Roy, 1998b), as well as a 5' RNA triphosphatase activity (Costas *et al.*, 1998). Methyltransferase activity, also associated with this protein, indicates that the complete mRNA capping reaction is associated with this single protein (Ramadevi *et al.*, 1998).

VP6

VP6 is encoded by RNA segment 9 and is a minor component of BTV inner cores (Roy, 1989; Roy *et al.*, 1990a). Roy *et al.* (1990a) demonstrated that the protein binds to viral ssRNA, dsRNA as well as DNA. VP6 contains a motif common to helicases (Stäuber *et al.*, 1997) and may therefore be responsible for unwinding the dsRNA prior to initiation of transcription and replication. In addition, the protein has ATPase activity, which may provide energy for the unwinding process (Stäuber *et al.*, 1997). Although VP6 is localized to the cytoplasm of infected cells, it possesses nuclear targeting signals, the significance of which is not yet clear (Yi *et al.*, 1996).

1.5.2 Non-structural proteins

Four different non-structural proteins have been identified. The two major non-structural proteins, NS1 and NS2, are synthesized abundantly in infected cells, whereas the two minor and closely related proteins, NS3 and NS3A, are barely detectable.

NS1

NS1 is encoded by RNA segment 6 and is synthesized in very large amounts in infected cells where it accumulates as virus-specific tubule structures in the cytoplasm of BTV-infected cells (Huismans, 1979; Huismans and Els, 1983; Urakawa and Roy, 1988). The NS1 protein is rich in cysteine residues, suggesting that it has a highly ordered disulfide-bonded structure (Roy *et al.*, 1990b). In addition, the protein also possesses a number of very strong hydrophobic regions which may play a role in tubule formation (Roy *et al.*, 1990a). The function of these tubular structures is unknown, but they may be involved in

the transport of mature virus particles from the VIBs to the cell membrane whereafter the viruses are released from the infected cells (Hyatt and Eaton, 1988).

NS2

The NS2 protein is encoded by RNA segment 8. The synthesis of NS2 coincides with the appearance of granular viral inclusion bodies (VIBs) in the cytoplasm of infected cells (Lecatsas, 1968; Thomas *et al.*, 1990). The presence of NS2 in VIBs suggests that this protein is involved in BTV replication and/or assembly. In addition to NS2, these VIBs have also been shown to contain ssRNA, dsRNA, NS1 and both complete and incomplete viral particles (Eaton and Hyatt, 1988; Eaton *et al.*, 1990; Brookes *et al.*, 1993). The inclusion bodies are therefore recognized as the sites where viral assembly processes occur. NS2 displays ssRNA but not dsRNA-binding activity (Huismans *et al.*, 1987) and it could thus act in the selection and condensation of the ten mRNA species into precursor viral particles (Eaton *et al.*, 1988; Huismans and Basson, 1983; Thomas *et al.*, 1990; Theron *et al.*, 1994; Uitenweerde *et al.*, 1995). The NS2 protein also has nucleotidyl phosphatase (NTPase) activity (Taraporewala *et al.*, 2001), which may be important for generating energy used in the transport, sorting, and packaging of viral mRNAs. The RNA-binding domain of NS2 has been mapped to the N-terminus of NS2 (Theron *et al.*, 1996). Several studies have indicated that ssRNA-binding of NS2 is non-specific (Huismans *et al.*, 1987; Thomas *et al.*, 1990; Uitenweerde *et al.*, 1995), but studies by Theron and Nel (1997) have indicated that BTV NS2 may have a specific affinity for the 3' terminus of BTV mRNA. NS2 is the only virus-specified phosphoprotein and is known to be phosphorylated on serine residues only (Huismans *et al.*, 1987; Thomas *et al.*, 1990). Phosphorylation does not appear to be important in ssRNA-binding (Thomas *et al.*, 1990), but it does seem to down-modulate the RNA-binding activity (Theron *et al.*, 1994).

NS3/NS3A

NS3 and NS3A are encoded by RNA segment 10 and are synthesized in very small amounts in infected cells. NS3A is a truncated form of NS3 and the proteins are encoded from two in-phase overlapping reading frames on the NS3 gene (French *et al.*, 1989). They differ only with respect to ten additional amino acids present at the N-terminus of NS3 (Van Staden and Huismans, 1991). The NS3 proteins are integral membrane glycoproteins (Wu *et al.*, 1992) and are localized to the endoplasmic reticulum (ER) and

Golgi complex of the cytoplasm of infected cells. The NS3 proteins may be involved in the release of the virus from the infected cells (Stoltz *et al.*, 1996; Hyatt *et al.*, 1991).

Table 1.3: Function of bluetongue virus proteins

Bluetongue virus protein	Function and Properties	Location
VP1	RNA- dependent RNA polymerase.	Sub-core
VP2	Serotype determining antigen. Hemagglutinin protein, Involved in cellular attachment and virus entry.	Outer capsid
VP3	Organization and size of sub-core structure. RNA binding.	Sub-core
VP4	Guanylyltransferase. Methyltransferase. NTPase activity.	Sub-core
VP5	Release of core into the cytoplasm. Contributory role in the induction of neutralizing antibodies.	Outer capsid
VP6	Helicase. Binds viral ssRNA and dsRNA and other RNA. NTPase activity. Possesses nuclear target signals.	Sub-core
VP7	Cell entry. Serogroup-specific antigen.	Outer capsid
NS1	Tubule formation. Involved in viral morphogenesis.	Cytoplasm, forms tubules
NS2	Binds single-stranded RNA. Phosphoprotein. Formation of VIB. NTPase activity.	Cytoplasm, VIB
NS3	Release of virus. Integral membrane proteins. Glycoprotein.	Cell membranes

* Table modified from data supplied by Peter P. C. Mertens (The RNAs and Proteins of dsRNA Viruses: Edited by Peter. P. C. Mertens and Dennis H. Bamford)

http://www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/Orbivirus.htm

1.6 ORBIVIRUS MORPHOGENESIS

Virus binding to mammalian cells is mediated by the VP2 protein (Hassan and Roy, 1999). The specific cellular receptors for VP2 binding have not yet been identified, but it is likely that a glycoprotein and a second unknown protein may serve as co-receptors for infection (Hassan and Roy, 1999). Recently, VP7 has also been implicated in cellular attachment by binding to specific cellular receptors of *Culicoides* species (Tan *et al.*, 2001). After binding of the virus to the cell receptors at sites which are characterized by the presence of clathrin, the clathrin-coated membrane surface grows and eventually detaches from the cell surface to yield a coated vesicle, which loses the clathrin coat. Following fusion of these vesicles, large endocytic vesicles (endosomes) are formed which enter the cell cytoplasm through endocytosis (Eaton *et al.*, 1990). The outer capsid proteins (VP2 and VP5) are removed in the endosomes (Hyatt *et al.*, 1992), which causes destabilization of the vesicle membrane and allows penetration of newly uncoated core particles into the cytoplasm of infected cells. The core particles associate with intermediate filaments of the cell cytoskeleton whereafter transcription of the viral RNA occurs. The viral dsRNA is retained within the protein core that contains RNA polymerase, helicase, capping enzyme, guanylyltransferase and transmethylase to synthesize, cap and methylate mRNA copies of the viral genome segments. Following transcription, translation of the viral mRNA generates proteins that condense with RNA to form VIBs. The VIBs are the sites of virus assembly and contains structural (VP5, VP2, VP3 and VP7) and non-structural proteins (NS1 and NS2) as well as virus particles at different stages of morphogenesis. VP2 is added to the BTV particles at the periphery of the VIBs after which the progeny virus particles are released into the cytoplasm of infected cells (Brookes *et al.*, 1993). Once these structures have been formed, the packaged ssRNA is copied to form dsRNA. The mechanism whereby BTV is released from infected cells has been the subject of some speculation. The intermediate filaments may provide a route whereby the progeny viruses are transported to the regions of the plasma or intracellular membranes containing NS3. The viruses can then be released from the infected cells by budding (Hyatt *et al.*, 1989). In mammalian cells, replication of orbiviruses leads to shut-off of host protein synthesis and contributes to cell lysis. Viruses are released both as enveloped particles by budding through the plasma membrane and as non-enveloped particles by extrusion through the membrane (Hyatt *et al.*, 1989).

1.7 RNA BINDING

The mechanism whereby individual orbivirus genomic segments are selectively recognized, bound and assembled into mature viral particles has yet to be determined. This mechanism must be precise, achieving a particle-to-infectivity ratio as high as 1.0. In order to be infectious, a BTV particle must contain a single copy of each one of the ten genomic RNA segments. Each segment must be recognized and a “memory” must develop which will ensure that the segments are not incorporated twice. The lack of free pools of non-packaged dsRNA in infected cells has led to the assumption that the viral mRNA is encapsidated with the minus strands subsequently being made using the packaged positive strands as templates. Single-stranded RNA-binding proteins may thus play an important role in the encapsidation process. For such proteins to fulfil this role, they should have both a specific and non-specific ssRNA-binding capability. Non-specific RNA-binding may be required for a condensing activity whereby the ssRNA segments can be localized in one place prior to being packaged. However, there must also be a degree of specificity in the RNA-protein interaction to ensure that in addition to only viral mRNA being packaged, only one of each of the different mRNA transcripts is packaged. Therefore, each viral segment may contain at least two operational recognition sequence domains, one that specifies that it is a viral and not cellular RNA, and a second that specifies that it is a specific RNA segment.

For BTV it has been shown that the VP6, NS2, VP3 and VP1 proteins all bind nucleic acids. The binding activity of VP6 has been shown to be non-specific (Roy, 1990a) and it binds ssRNA, dsRNA and DNA. VP3 has been reported to bind ssRNA in a sequence-independent manner and is also capable of binding to dsRNA and DNA (Loudon *et al.*, 1992). Several studies have shown that the NS2 protein binds viral mRNA (Huismans *et al.*, 1987), cellular ssRNA (Huismans *et al.*, 1987) and poly (U)-oligonucleotides (Huismans and Basson, 1983; Uitenweerde *et al.*, 1995), but it does not bind to dsRNA (Huismans *et al.*, 1987). NS2 was proposed to be involved in the packaging of the ten different orbiviral mRNA segments into precursor viral particles because it only binds to ssRNA. Amino acid sequence analysis between orbivirus NS2 and cognate reovirus and rotavirus ssRNA-binding proteins indicated a N-terminal conserved amino acid motif (Van Staden *et al.*, 1991b). The role of this motif in RNA-protein binding was investigated and it was subsequently shown that it is required for maintaining the conformation of the NS2 protein in such a manner so as to allow RNA binding (Theron *et al.*, 1996).

Conserved 5' and 3' hexanucleotide sequences (Rao *et al.*, 1983; Mertens and Sanger, 1985) as well as short terminal segment-specific inverted repeats (Cowley *et al.*, 1992) have been reported to be present on all ten BTV mRNA segments. Both these sequences may play a role in BTV morphogenesis by possibly serving as signals to ensure that only one copy of each mRNA segment is packaged per virus particle. Results published by Theron and Nel (1997) indicated that the 5' and 3' terminally conserved hexanucleotide sequences of BTV are not directly involved in RNA-NS2 interactions and that the full-length BTV segment 8 mRNA transcripts are necessary for optimal binding. RNA-protein binding was further characterized by competition experiments with radioactively labelled BTV RNA and other homologous and heterologous ssRNA (Theron and Nel, 1997). The various ssRNA all interacted with the BTV NS2 protein, but different binding affinities were observed.

RNA-protein binding interactions in other members of the family *Reoviridae* have also been studied. The reovirus protein, σ NS, binds to single-stranded viral mRNA (Huimans and Joklik, 1976). In rotaviruses, there are various proteins that exhibit RNA-binding activity (Patton, 1995). The NSP1 protein displays affinity for the 5' end of rotavirus mRNA (Hua *et al.*, 1994) and may include residues located within the first 34 nucleotides. NSP2 has an affinity for all RNA, not necessarily virus-specific (Kattoura *et al.*, 1992), whilst VP1 displays a binding affinity for the 3' end of the viral RNA (Patton, 1995). VP2 (Boyle and Holmes, 1986) and NSP3 (Mattion *et al.*, 1992) both bind to ssRNA and dsRNA. NSP3 binds to a specific consensus sequence, GACC, present at the 3' terminal end of all rotavirus mRNAs and mutation of this sequence affects the binding affinity of NSP3 to the RNA (Poncet *et al.*, 1994). This selective binding ability may allow NSP3 to select rotavirus mRNA among other cellular RNA. NSP3 and σ NS are functional analogues of the BTV NS2 protein. It may be that BTV NS2 is responsible for the selection and packaging of the ten different RNA segments of BTV.

Studies performed on influenza virus, a negative-sense segmented RNA virus, suggested that the structure of the RNA is very important in RNA-protein binding interactions. The NS1 protein of this virus displays RNA binding (Hatada *et al.*, 1992). The terminal regions of the RNA exist as the stem of a panhandle secondary structure and a mismatched adenylate residue in this panhandle motif is crucial for RNA-protein binding (Hatada *et al.*, 1997). In the dsRNA virus, *Saccharomyces cerevisiae* (ScVL1), a

region on the RNA was recognized as a binding site in RNA-protein binding required for encapsidation of the viral RNA (Yao *et al.*, 1997). It is a minimum of 20 bases long and consists of a stem interrupted by a bulged A residue with a central loop of several bases. Secondary structure analysis of the BTV mRNA transcripts predicted base-pairing at the inverted terminal repeat sequences which results in 5'- and 3'- terminal overhangs as well as segment-specific stem-loop structures (Cowley *et al.*, 1992). Such secondary structures may mediate assortment of the different viral mRNAs, by acting as packaging signals for the respective mRNA segments. A protein present in cells infected with wound tumor virus (WTV) was identified that binds WTV transcripts in a structure-dependant manner (Dall *et al.*, 1990). Deletion of the 5' or 3' inverted repeat sequences resulted in a disruption of basepairing of inverted repeat sequences. This deletion led to a disruption of the secondary structure of the RNA and a decline in RNA-protein binding.

1.8 AIMS OF THIS STUDY

From the preceeding sections, it is evident that the mechanism whereby genomic segments are selected, assorted and packaged into orbivirus particles remains unknown. Although the NS2 protein is capable of binding to ssRNA, the interaction appears to be non-specific. Since all ten BTV RNA segments contain inverted repeat sequences, it was proposed that these sequences might play a role in RNA-NS2 binding interactions by forming higher-order structures which may be unique for each of the different RNA segments. It is thus possible that the answer to specificity in the RNA-NS2 recognition process may involve the different secondary structures of the individual orbivirus genomic segments.

Therefore, the aims of this investigation were the following:

- To investigate the importance of the BTV mRNA inverted repeat sequences in RNA-NS2 interactions using mutant transcripts lacking these sequences in nitrocellulose-binding assays (Chapter 2).
- To investigate the use of an IAsys biosystem for quantification of RNA-NS2 binding using different orbivirus segment 8 mRNA transcripts (Chapter 3).

- To identify RNA secondary structures that may be required in RNA-NS2 binding using computational analysis (Chapter 4).

CHAPTER 2:
THE IMPORTANCE OF THE 5' AND 3' TERMINAL INVERTED REPEAT SEQUENCES
WITHIN BLUETONGUE VIRUS mRNA FOR INTERACTION WITH THE VIRUS NON-
STRUCTURAL PROTEIN NS2

2.1 INTRODUCTION

The genome of BTV, the prototype orbivirus, consists of ten segments of double-stranded RNA. Although morphogenesis of orbiviruses entails a process whereby each precursor viral particle acquires a single copy of each of the ten dsRNA genome segments, the process whereby the virus can successfully select, assort and package each of the ten genomic segments into precursor viral particles has yet to be elucidated. The lack of free pools of double-stranded RNA in BTV-infected cells suggests that this selection must occur on a single-stranded RNA level. Thus, single-stranded RNA-binding proteins may play an important role in this process.

The NS2 protein of BTV, encoded by RNA segment 8, has been reported to bind ssRNA (Huismans *et al.*, 1987; Thomas *et al.*, 1990; Uitenweerde *et al.*, 1995). Comparative sequence analysis between different orbivirus NS2 proteins and the cognate ssRNA-binding proteins of reo- and rotaviruses has led to the identification of a conserved N-terminal nine amino acid motif (Van Staden *et al.*, 1991b). The functional significance of this motif in NS2-ssRNA-binding was investigated by analysing the binding activity of different site-directed mutant NS2 proteins. The results obtained indicated that the amino acid motif might be important for maintaining the conformation of the NS2 protein such that it is capable of binding to ssRNA (Theron *et al.*, 1996). However, studies relating to the binding of NS2 to ssRNA have reported only non-specific interactions (Huismans *et al.*, 1987; Huismans and Basson, 1983; Uitenweerde *et al.*, 1995). Thus, if NS2 does play a role in mediating assortment and packaging of the individual genomic segments, it is possible that the signals required for specific RNA-protein interactions may be localized in each of the ten viral mRNA species.

Nucleic acid sequence analysis of the BTV mRNA segments has revealed that all ten RNA segments contain conserved hexanucleotide sequences at both the 5' and 3' termini of each segment (Rao *et al.*, 1983). Deletion of these conserved hexanucleotide sequences has been reported not to influence binding of the mutant mRNA transcripts to NS2 (Theron and Nel, 1997). In addition to the conserved hexanucleotide sequences, the BTV RNA segments also possess segment-specific inverted repeat sequences adjacent to the conserved hexanucleotide sequences (Cowley *et al.*, 1992). Considering the likely importance of specificity in the RNA-protein binding process required for encapsidation of the different viral mRNAs, these conserved configurations provide

circumstantial evidence for some functional role in the encapsidation process. Secondary structure analysis of the BTV mRNA transcripts predicted base-pairing at the inverted repeat sequences which resulted in 5' and 3' terminal overhangs as well as segment-specific stem-loop structures (Cowley *et al.*, 1992). These sequences may therefore have a direct or indirect influence on RNA-NS2 binding by being responsible for maintaining the mRNA structure required.

In this part of the study the importance of the inverted repeat sequences in the ability of the RNA to interact with BTV NS2 was investigated. Using PCR-based methodology, deletions were introduced into cDNA copies of RNA segment 8 whereby the 5' and/or 3' inverted repeat sequences were removed, and the effect of these deletions on RNA-NS2 binding was subsequently investigated by nitrocellulose-binding assays.

2.2 MATERIALS AND METHODS

2.2.1 Deletion mutagenesis of the NS2-encoding gene – Preparation of mutant NS2 genes

2.2.1.1 PCR-based deletion of the segment-specific inverted repeat sequences

Table 2.1: Sequences of the PCR primers used to construct mutant NS2 genes lacking inverted repeat sequences

Primer	Polarity	Oligonucleotide sequence	Reference
DINS2 (-)	-	CGAGGATCCTTGAGTCATGGAGCA	This study
DINS2 (+)	+	CGAGGATCCCCCCTAACCACGC	This study
BTVm3'	-	CGAGGATCCGTAAAATCCCCCCTAA	Theron and Nel, 1997
BTVm5'	+	CGAGGATCCAAATCCTTGAGTCAT	Theron and Nel, 1997

* The *Bam* HI sites incorporated into the primers are underlined

Deletion mutants of the BTV segment 8 gene lacking the 5' and/or 3' inverted repeat sequences were constructed using a PCR-based method and different combinations of

116076242
615432361

the primers as indicated in Fig. 2.1. *Bam* HI restriction sites were incorporated into the primers during their synthesis. A plasmid (pBS.BTVS8, Dr. J. Theron, Department of Microbiology and Plant Pathology, University of Pretoria), containing the full-length BTV-10 NS2 gene cloned in the *Bam* HI site of pBS (Stratagene), was used as a template in each of the reactions. The PCR reaction mixture contained 10 μ l of a 10 $\times$ Taq polymerase buffer (500 mM KCl; 100 mM Tris-HCl (pH=9); 1.0 % Triton X-100), 6 μ l 25 mM $MgCl_2$, 1 μ l of a 25 mM deoxynucleotide triphosphate (dNTP) mixture, 25 ng plasmid template (pBS.BTVS8), 100 pmol of each of the appropriate primers and 2 U of Taq DNA polymerase (Promega). The reaction mixture was adjusted to 100 μ l with UHQ water and overlaid with paraffin oil. The samples were subjected to 30 cycles of amplification at 94 $^{\circ}C$ for 2 min, 68 $^{\circ}C$ for 1 min and 72 $^{\circ}C$ for 2 min in a Hybaid Omnigene thermocycler. As a control, an identical reaction mixture was prepared except no template DNA was added.

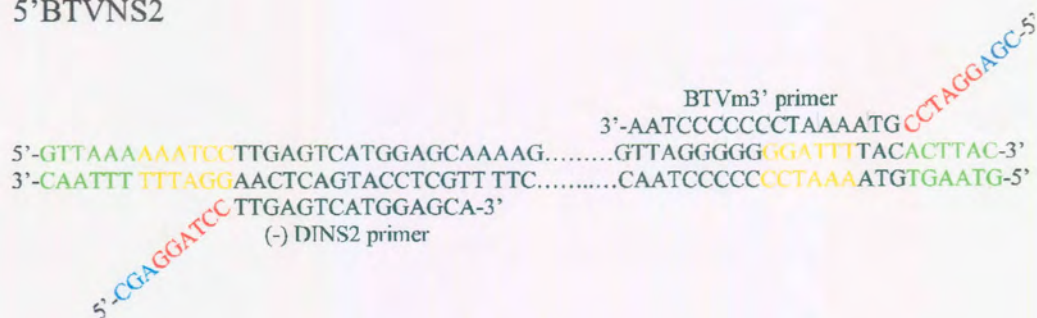
2.2.1.2 Agarose gel electrophoresis

The amplicons were visualized by agarose gel electrophoresis. Horizontal 1 % (w/v) agarose slab gels were cast and electrophoresed at 100 V in 1 $\times$ TAE buffer (40 mM Tris-HCl (pH=8.5); 20 mM NaOAc; 1 mM EDTA) using a submarine agarose gel electrophoresis unit from Hoefer. The agarose gels were stained with ethidium bromide (0.5 μ g/ml) and the DNA fragments were visualized by UV fluorescence on a transilluminator. The appropriate DNA molecular weight marker was included on the gel.

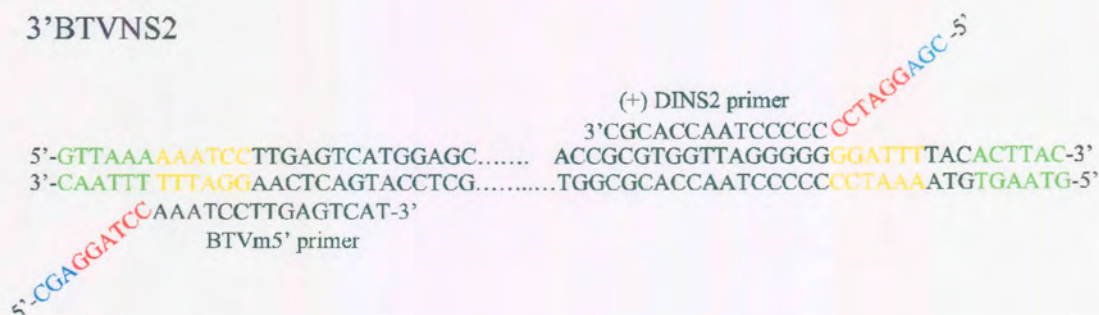
2.2.1.3 Concentration of the PCR products

To concentrate the PCR products, two reactions with a reaction volume of 100 μ l each, were pooled and the volume was adjusted to 300 μ l with ddH₂O. An equal volume of chloroform (300 μ l) was added and the mixture was mixed gently. After centrifugation for 1 min, the upper layer was removed. A tenth of the volume of 3 M NaOAc (pH=7) and 2.5 volumes absolute ethanol were added. After precipitation for 1.5 h at -20 $^{\circ}C$, the pellet was collected by centrifugation at 4 000 rpm for 10 min in a Hettich Mikro-12 rotor. The pellet was washed three times with 70 % ethanol and vacuum dried. The dried pellet was resuspended in 35 μ l 1 $\times$ TE (10 mM Tris-HCl (pH=8.0); 1 mM EDTA) and analyzed by agarose gel electrophoresis (Section 2.2.1.2).

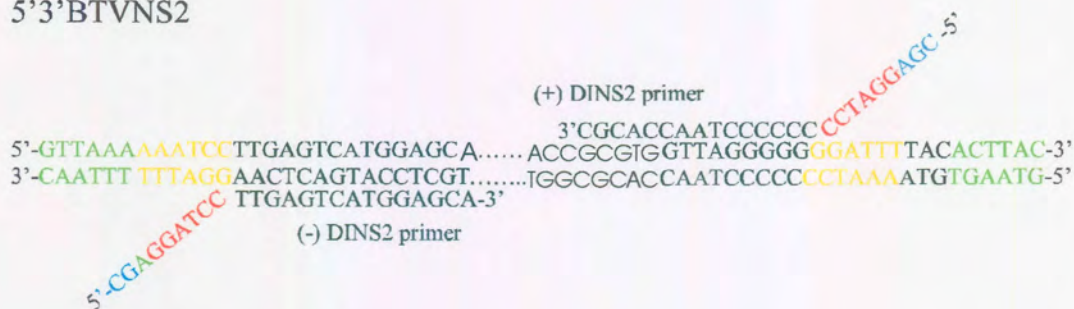
5'BTVNS2



3'BTVNS2



5'3'BTVNS2



-  BTV segment 8 hexanucleotide sequences
-  BTV segment 8 inverted repeat sequences
-  *Bam* HI restriction enzyme sites
-  Additional sequences at the end of the primers
-  BTV segment 8 sequences

Fig. 2.1: Schematic representation of the primers and annealing sites used during the construction of different NS2 genes lacking inverted repeat sequences.

2.2.1.4 Restriction enzyme digestion of PCR amplicons

Restriction enzyme digestion of the amplicons was performed with *Bam* HI (Promega). The appropriate salt concentration was obtained by using the 10 × buffer B (6 mM Tris-HCl (pH=7.5); 6 mM MgCl₂; 50 mM NaCl; 1 mM DTT) supplied by the manufacturer. The amplicons (1 µg of 5'BTVNS2, 3'BTVNS2 and 5'3'BTVNS2) were digested with 1 U of the *Bam* HI enzyme in a final reaction volume of 15 µl at 37 °C for 1.5 h. After incubation, the DNA fragments were analyzed by agarose gel electrophoresis (Section 2.2.1.2). A molecular weight marker (Promega: Lambda DNA restriction enzyme digested with *Eco* RI + *Hind* III) was used to estimate the size of the respective DNA restriction fragments.

2.2.1.5 Purification of restriction enzyme digested PCR fragments

Restriction fragments were purified from agarose gels by centrifugation of the gel slice, containing the desired DNA fragment, through siliconized glass wool (Heery *et al.*, 1990). For this purpose, a 0.5 ml tube was punctured and plugged with siliconized sterile glass wool. The 0.5 ml tube, containing the gel slice, was placed into a 1.5 ml tube and centrifuged at 15 000 rpm for 5 min in a Hettich mikro-12 rotor. The glass wool blocks the passage of agarose, but DNA is allowed to pass through the holes in the tube. The eluate containing the DNA was collected in the bottom tube and the recovery of the purified DNA fragments was analyzed by agarose gel electrophoresis (Section 2.2.1.2).

2.2.2 Deletion mutagenesis of the NS2-encoding gene – Preparation of vector DNA

2.2.2.1 Preparation of competent cells and transformation

The technique as described by Sambrook *et al.* (1989) was used, with the following slight modifications. A single colony of *E. coli* JM109 bacterial cells (Promega) was inoculated into 3 ml of LB broth (1 % tryptone; 0.5 % yeast extract; 1 % NaCl; pH=7.4) and incubated overnight with agitation at 37 °C. One ml of the overnight culture was used to inoculate 100 ml pre-warmed LB broth and the cells were incubated at 37 °C until an optical density (OD) at 550 nm of 0.35 to 0.5 was reached. The cells were incubated on ice for 30 min and then collected from 20 ml of culture by centrifugation at 4000 rpm for 10 min at 4 °C in a pre-cooled Sorvall HB 4 rotor. The cells were resuspended in 10 ml sterile ice-cold 100 mM CaCl₂, incubated on ice for 2 h and centrifuged for 10 min at 4000 rpm in pre-cooled HB-4 rotor. The cells were gently resuspended in 1 ml ice-cold CaCl₂ and incubated on ice for 3 h before use.

The competent cells were transformed with the pBS vector (Fig. 2.2; Stratagene) using the heat shock method (Sambrook *et al.*, 1989). Competent cells (200 μ l) were mixed with 25 ng of the pBS plasmid DNA. After leaving the mixture for 30 min on ice, the cells were heat-shocked for 90 s at 42 °C and immediately cooled on ice for 2 min. Pre-warmed LB broth (800 μ l) was added to the transformation mixture and then incubated with agitation for 1 h at 37 °C. The cells were plated in aliquots of 200 μ l onto a LB agar plate supplemented with ampicillin (50 μ g/ml) and incubated at 37 °C for 16 h. To determine the transformation efficiency, pUC 18 plasmid DNA (Roche), with a known concentration, was also transformed. To test for contamination, competent cells only were plated onto the LB agar plates supplemented with ampicillin.

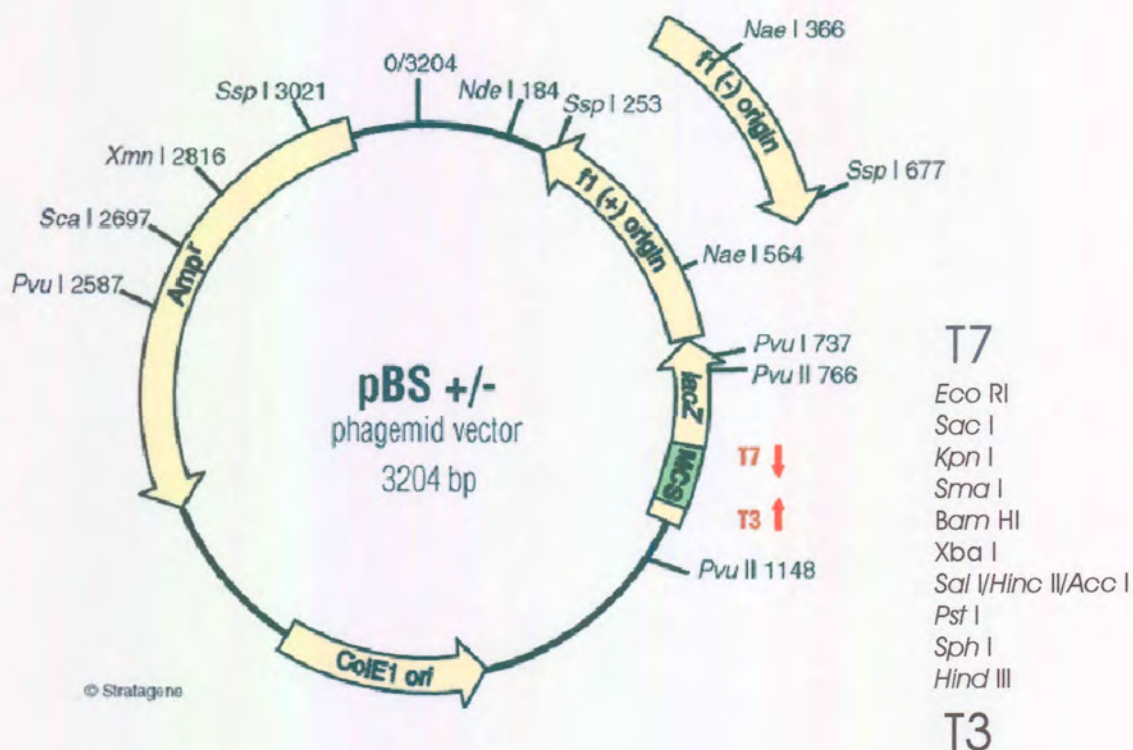


Fig. 2.2: Plasmid map of the pBS vector (Stratagene) used during this study as a cloning and transcription vector.

2.2.2.2 Small-scale plasmid DNA extraction

Plasmid DNA was isolated using the alkaline lysis method as described by Sambrook *et al.* (1989). A bacterial colony containing pBS (Stratagene) was inoculated into 3 ml LB broth (1 % tryptone; 0.5 % yeast extract; 1 % NaCl; pH=7.4) containing ampicillin (50 µg/ml) and incubated with agitation for 16 h at 37 °C. The cells were harvested from the overnight culture by centrifugation for 1 min at 15 000 rpm in a Hettich Mikro-12 rotor and resuspended in 100 µl Solution 1 (50 mM glucose; 10 mM EDTA; 25 mM Tris-HCl, pH=8). The cells were lysed and the DNA denatured following the addition of 200 µl Solution 2 (0.2 M NaOH; 1 % SDS). After incubation on ice for 5 min, 150 µl Solution 3 (3 M NaOAc, pH=4.8) was added and the mixture was incubated on ice for 15 min. The chromosomal DNA was removed by centrifugation at 15 000 rpm for 10 min in a Hettich Mikro-12 rotor. The plasmid DNA, in the supernatant, was precipitated with 2.5 volumes absolute ethanol for 1 h at -20 °C. After centrifugation for 10 min at 15 000 rpm in a Hettich Mikro-12 rotor, the pellet was washed with 70 % ethanol and resuspended in 30 µl 1 × TE. The extracted plasmid DNA was analyzed by agarose gel electrophoresis on a 0.8 % agarose gel (Section 2.2.1.2).

2.2.2.3 Purification of plasmid DNA

Plasmid DNA obtained from small-scale plasmid DNA extractions (Section 2.2.2.2) was incubated with 1 µl RNase A (10 mg/ml) (Roche) at 37 °C for 1 h. The RNase A was boiled at 95 °C for 5 min before use to inactivate DNases that may be present in the enzyme preparation. The DNA was precipitated by the addition of 30 µl of 20 % PEG 6000 in the presence of 2.5 M NaCl (Sambrook *et al.*, 1989). The mixture was incubated on ice for 20 min and then centrifuged at 15 000 rpm in a Hettich Mikro-12 rotor for 20 min. The pellet was washed with 70 % ethanol, vacuum-dried and resuspended in 25 µl 1 × TE. Plasmid DNA of sufficient purity for digestion by restriction enzymes was obtained.

2.2.2.4 Restriction enzyme digestion of vector DNA

The pBS plasmid DNA was restriction enzyme digested with *Bam* HI (Promega). The appropriate salt concentration was obtained by using the 10 × buffer B supplied by the manufacturer. The pBS plasmid (1 µg) was cut with 1 U of *Bam* HI enzyme. Buffer B

(1.5 μ l) was added and the final reaction volume was 15 μ l. The reactions were incubated at 37 °C for 1.5 h. After incubation, the restriction fragments were analyzed using agarose gel electrophoresis (Section 2.2.1.2). An uncut pBS plasmid (Stratagene) was included on the agarose gel to determine if restriction enzyme digestion did occur.

2.2.2.5 Purification of restriction enzyme digested DNA fragments

The restriction enzyme digested pBS vector was purified from an agarose gel by an alternative, inexpensive method (Boyle and Lew, 1995). The linearized plasmid DNA fragment was excised from a low melting temperature agarose gel and transferred to a 1.5 ml tube. Two volumes of 6 M NaI were added and the gel slice was incubated at 55 °C for 5 min. The appropriate volume (5 μ l) of silica (100 mg/ml silica (Sigma) in 3 M NaI) was added and the mixture incubated on ice for 10 min. The silica was pelleted by centrifugation at 10 000 rpm in a Hettich Mikro-12 rotor for 1 min. After the pellet was washed four times with 0.5 ml of wash buffer (50 mM NaCl; 10 mM Tris-HCl (pH=7.5); 2.5 mM EDTA; 50 % v/v ethanol), the DNA was eluted in 10 μ l of ddH₂O. The purified DNA fragment was analyzed by agarose gel electrophoresis (Section 2.2.1.2).

2.2.3 Cloning of PCR fragments into pBS

2.2.3.1 Ligation

Ligation reactions were prepared for each of the respective amplicons (i5'BTVNS2, i3'BTVNS2 and i5'3'BTVNS2). Each reaction mixture (10 μ l) contained 1 U T4 DNA ligase (Roche), 1 μ l of a 10 $\times$ ligation buffer (660 mM Tris-HCl; 10 mM DTT; 50 mM MgCl₂; 10mM ATP; pH=7.5), 150 ng linearized pBS and 450 ng of the restriction enzyme digested amplicon. The ligation mixtures were incubated for 16 h at 15 °C.

2.2.3.2 Preparation of competent cells and transformation

Competent *E. coli* JM109 (Promega) cells were prepared as described in Section 2.2.2.1. Half the volume of the ligation reaction mixture (5 μ l) was added to a glass tube containing 200 μ l of competent cells. The cells were incubated on ice for 30 min and heat-shocked for 90 s at 42 °C. After cooling the cells on ice for 2 min, 800 μ l pre-warmed LB broth was added to the tube and the mixture was incubated with agitation for 1 h at 37 °C. The mixtures were plated in aliquots of 200 μ l onto LB agar plates containing ampicillin (50 μ g/ml) together with 10 μ l of 100 mM IPTG and 50 μ l of X-Gal

(2 % w/v), and then incubated at 37 °C for 16 h. The transformation efficiency was calculated from cells transformed with pUC 18 plasmid DNA (Roche) of a known concentration. To test for contamination, competent cells only were plated onto the selective LB agar plates.

2.2.4 Selection of recombinants

The pBS vector contains a multiple cloning site within the α -peptide coding region of β -galactosidase. Insertional inactivation of the α -peptide allows for the direct identification of recombinant clones by blue/white selection on indicator plates. The white colonies were selected as putative recombinants, grown overnight in LB broth supplemented with ampicillin and used for further characterization.

2.2.4.1 Plasmid DNA extractions

Small-scale DNA isolations were performed on putative recombinants (white colony) as well as a control non-recombinant (blue colony) as previously described in Section 2.2.2.2, after which the plasmid DNAs were analyzed by agarose gel electrophoresis (Section 2.2.1.2). Plasmid DNA isolated from a blue colony served as a marker to identify plasmids that were larger in size (recombinant) based on their slower migration in agarose gels.

2.2.4.2 Restriction enzyme digestions

Putative recombinant plasmid DNA was treated with RNase A (Section 2.2.2.3) and then subjected to restriction enzyme digestion to confirm that they contained the cloned insert DNA. The plasmid DNA was thus cut with *Bam* HI (Promega) for 1 h at 37 °C (Section 2.2.1.4). Restriction enzyme digestion was also used to determine whether the cloned insert DNA was under the transcriptional control of the T7 RNA polymerase promoter. The appropriate salt concentration was obtained by using the supplied 10 × buffer J (10 mM Tris-HCl; 7 mM MgCl₂; 50 mM KCl; 1 mM DTT; pH=7.5). The recombinant plasmid DNA (1 µg) was cut with 1 U of *Sma* I enzyme (Promega) in a final reaction volume of 15 µl. The reactions were incubated at 25 °C for 1.5 h. The restriction enzyme digested fragments were analyzed by agarose gel electrophoresis (See Section 2.2.1.2) and uncut plasmid DNA was included as a control to determine if restriction had indeed occurred.

2.2.4.3 Nucleic acid sequencing of mutant NS2 genes

2.2.4.3.1 Preparation of plasmid DNA

Large-scale plasmid DNA extractions were performed using the alkaline lysis method as described by Sambrook *et al.* (1989), with the following modifications. Bacterial colonies containing plasmids pBS.i5'BTVNS2, pBS.i3'BTVNS2 or pBS.i5'3'BTVNS2, were inoculated into 200 ml LB broth containing ampicillin (50 µg/ml). The cells were collected by centrifugation at 7 000 rpm in a Sorvall S10 rotor for 10 min and resuspended in 10 ml Solution 1 (50 mM glucose; 10 mM EDTA; 25 mM Tris-HCl; pH=8). After incubation on ice for 30 min, 20 ml Solution 2 (0.2 M NaOH; 1 % SDS) was added. After incubation on ice for 5 min, 15 ml 3 M NaOAc (pH=4.8) was added and incubation was continued on ice for 1 h. After centrifugation for 20 min at 20 000 rpm in a Sorvall S10 rotor, the supernatant was filtered through Whatman filter paper and the plasmid DNA precipitated by the addition of 2 volumes of 100 % ethanol at –20 °C for 1 h. The precipitate was collected by centrifugation at 10 000 rpm in a Sorvall S10 rotor for 20 min and the pellet resuspended in 12 ml 1 × TE. To precipitate the RNA, 5 ml 7.5 M ammonium acetate was added and incubated on ice for 30 min. The RNA precipitate was removed by centrifugation at 10 000 rpm in a Sorvall S10 rotor for 10 min and the DNA in the supernatant precipitated with 2 volumes of 100 % ethanol at – 20 °C for 1 h. After centrifugation at 10 000 rpm in a Sorvall S10 rotor for 10 min, the pellet was washed with 70 % ethanol to remove the residual salt. The pellet was dried in a vacuum dryer and resuspended in 500 µl ddH₂O. Plasmid DNA was purified by RNase A-PEG purification as previously described (Section 2.2.2.4).

2.2.4.4.2 Sequencing reaction

The different constructs (pBS.i5'BTVNS2, pBS.i3'BTVNS2 and pBS.i5'3'BTVNS2) were sequenced using the ABI Prism™ Big Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer) together with either the M13 forward (5'-GTTTCCCAGTCACGAC-3') or M13 reverse (5'-GTAAAACGACGGCCAGT-3') sequencing primers (Roche). The primer (1.8 pmol) was annealed to 500 ng of template DNA in a 10 µl reaction volume containing 4 µl of the terminator ready reaction mixture. Cycle sequencing (25 cycles) was performed in a GeneAmp 2400 PCR thermocycler using the following cycling parameters: 96 °C for 30 s, 50 °C for 15 s, 60 °C for 4 min.

2.2.4.4.3 Purification of extension products

The extension products were purified according to procedures described by the manufacturer of the sequencing kit (Perkin-Elmer). The entire 10 μ l of the sequencing reaction was transferred into a 1.5 ml tube and 2 μ l 3 M NaOAc (pH=4.6) and 50 μ l 95 % ethanol were added. The reaction mixture was vortexed and incubated on ice for 10 min. The DNA was pelleted by centrifugation at 15 000 rpm for 30 min and the supernatant discarded. The pellet was washed three times with 70 % ethanol and dried in a vacuum dryer.

2.2.4.4.4 Gel electrophoresis of purified extension products

The sequencing reaction was resuspended in 6 μ l loading buffer (deionized formamide; 25 mM EDTA (pH=8); 50 mg/ml blue dextran in a ratio of 5:1 formamide to EDTA/Blue dextran). The sample was heated to 90 °C for 2 min and then loaded onto an ABI PRISM 377 automated sequencer. The obtained sequences were analyzed using the Sequence Navigator software program (Perkin-Elmer).

2.2.5 Preparation of radioactively labelled RNA transcripts

2.2.5.1 Linearization of recombinant plasmid DNA

RNA transcripts were prepared by run-off transcription following linearization of the recombinant transcription vectors. Recombinant plasmids (pBS.i5'BTVNS2, pBS.i3'BTVNS2 and pBS.i5'3'BTVNS2) as well as a plasmid containing the full-length segment 8 gene of BTV (pBS.BTVS8) (Uitenweerde *et al.*, 1995) and a plasmid containing the segment 8 gene of which the 5' and 3' conserved hexanucleotide sequences have been deleted (pBS. Δ mBTVNS2) (Theron and Nel, 1997), were linearized with *Xba* I (Promega). The plasmids (1 μ g) were cut with 1 U of *Xba* I (Promega) in 10 $\times$ restriction enzyme buffer D (6 mM Tris-HCl; 6 mM MgCl₂; 150 mM NaCl; 1 mM DTT; pH=7.9), as supplied by the manufacturer, for 1 h at 37 °C in a final reaction volume of 15 μ l.

2.2.5.2 Purification of linearized DNA fragments

After restriction enzyme digestion, the samples were analyzed by agarose gel electrophoresis (Section 2.2.1.2). The linearized DNA fragments were excised from the

agarose gel and purified using the modified glassmilk method as described in Section 2.2.2.5.

2.2.5.3 In vitro transcription

Radiolabelled RNA was synthesized in 20 μ l reaction mixtures containing 40 mM Tris-HCl (pH=7.5); 6 mM $MgCl_2$; 10 mM NaCl; 2 mM spermidine; 10 mM dithiothreitol; 0.5 mM ribonucleotides (ATP, GTP, UTP); 12 μ M CTP; 50 μ Ci of [α - ^{32} P] CTP, 0.5 μ g of linearized template; 20 U RNasin (Promega) and 20 U T7 RNA polymerase (Promega). The reaction mixtures were incubated at 37 °C for 1.5 h. Template DNA was destroyed by treatment with 10 U RNase-free DNase (Roche) for 15 min at 37 °C. The integrity of the transcripts was analyzed on an agarose gel (Section 2.2.1.2).

2.2.5.4 Precipitation of the single-stranded RNA

To remove unincorporated ribonucleotides, the RNA was precipitated (Sambrook *et al.*, 1989). Three volumes of 100 % ethanol and 0.1 volume of 5 M ammonium acetate were added to 40 μ l of the RNA transcription reaction mixture. The reaction mixture was incubated on ice for 1 h and centrifuged at 15 000 rpm in a Hettich Mikro-12 rotor for 20 min at 4 °C. After the pellet was washed in 70 % ethanol, it was resuspended in 20 μ l DEPC (1 % v/v diethylpyrocarbonate) water. The quality of the RNA transcripts was analyzed on an agarose gel and visualized by ethidium bromide staining (Section 2.2.1.2). An aliquot of the precipitated RNA was also counted in a scintillation counter to determine the efficiency of radiolabel incorporation.

2.2.6 Expression of the BTV NS2 protein

2.2.6.1 Cells and viruses

The construction of a recombinant baculovirus, expressing the BTV NS2 protein, has been described previously (Uitenweerde *et al.*, 1995). *Spodoptera frugiperda* cells (Summers and Smith, 1987) were propagated in suspension at 27 °C in Grace's insect medium (Highveld Biological) containing 10 % fetal calf serum (FCS) and the appropriate antibiotics.

Monolayers of *Sf* cells used for infections were obtained by seeding an 80 cm² tissue culture flask with 2.5×10^6 cells. The cell density was determined with a haemocytometer and the viability of the cells was determined by staining the cells with

trypan blue (0.4 % trypan blue in 1 × PBS (137 mM NaCl; 2.7 mM KCl; 4.3 mM Na₂HPO₄·2H₂O; 1.4 mM KH₂PO₄; pH=7.4). Following attachment for 1 h at room temperature, the medium was removed and the virus suspension was added to the cells at a MOI of 5 pfu/ml. After incubation for 1 h at room temperature, to allow the virus to adsorb to the cells, the virus suspension was removed and fresh Grace's insect medium containing serum and antibiotics (Highveld Biological) was added. The tissue culture flasks were incubated at 27 °C for 36 h and the monolayers were examined daily. Uninfected *Sf* cells were included as a control.

2.2.6.2 Virus titration

The virus titer was determined according to a method described by Possee and Howard (1987). *Spodoptera frugiperda* cells were seeded in 35 mm tissue culture dishes at a density of 2×10^6 cells/well and allowed to attach for 1 h at room temperature. A dilution series of the virus stock was prepared and added to the wells after the medium had been removed. The dishes were incubated at room temperature for 1 h to allow infection of the cells. Sterile 3 % low melting temperature agarose was melted and cells were overlayed with a 1:1 dilution of the agarose in Grace's insect medium (Highveld Biological) after removal of the diluted virus suspensions. The dishes were placed in a Tupperware container with wet towel paper and incubated for 4 days at 27 °C. After incubation, 1 ml neutral red (1 mg/ml in H₂O, diluted 10 × with Grace's insect medium) was added to each well and the plates were incubated for 5 h at 27 °C after which the neutral red was removed and the tissue culture dishes incubated overnight. Uninfected *Sf* cells were also included as a control.

2.2.6.3 Preparation of cell extracts

Mock-infected or infected *Sf* cells were harvested with a syringe and the cells concentrated by centrifugation at 2000 rpm for 15 min in a Hettich Mikro-12 rotor. The cells were washed twice in 1 × PBS (137 mM NaCl; 2.7 mM KCl; 4.3 mM Na₂HPO₄·2H₂O; 1.4 mM KH₂PO₄; pH=7.4) and resuspended in 1 ml ice-cold 0.01 M STE-TX (10 mM NaCl; 10 mM Tris-HCl; 1 mM EDTA; pH=7.4; 0.5 % Triton X-100). After 10 min on ice, the suspension was homogenized by 15 strokes with a dounce homogenizer. The nuclei were removed by centrifugation at 2 000 rpm in a Hettich Mikro-12 rotor for 5 min and the supernatants (S10 fraction) were stored at –70 °C until needed.

2.2.6.4 SDS-polyacrylamide gel electrophoresis

Samples were analyzed by SDS-PAGE as described by Laemmli (1970). Samples, prepared as described in Section 2.2.6.3 were mixed with an equal volume of 2 × protein solvent buffer (PSB; 125 mM Tris-HCl (pH=6.8); 4 % SDS; 20 % glycerol; 10 % 2-mercaptoethanol; 0.002 % bromophenol blue). The samples were boiled for 5 min and sonicated for 15 min before being loaded onto a SDS-PAGE gel.

For electrophoresis, a 12 % separating gel (acrylamide:bisacrylamide (30:0.8); 0.375 M Tris-HCl (pH8.8); 0.1 % SDS; 0.06 % ammonium persulphate; 0.6 % TEMED) and 5 % stacking gel (acrylamide:bisacrylamide(30:0.8); 0.125 M Tris-HCl (pH = 6.8); 0.1 % SDS; 0.06 % ammonium persulphate; 0.06 % TEMED) was used, and TGS (25 mM Tris-HCl; 250 mM glycine (pH=8.3); 0.1 % SDS) was used as the electrophoresis buffer. Electrophoresis was performed in a Hoefer Mighty Small TM electrophoresis unit for 2 h at 120 V. Gels were electrophoresed in duplicate and after electrophoresis, one gel was stained with Coomassie brilliant blue and the other unstained gel was used in a Western blot. Gels were stained with Coomassie brilliant blue (0.125 % Coomassie brilliant blue prepared in 50 % methanol; 10 % acetic acid) and then destained in destaining solution (30 % methanol; 10 % acetic acid) before being dried using a Hoefer gel dryer.

2.2.6.5 Western blot analysis

Western blot analysis was performed as described by Sambrook *et al.* (1989). After SDS-PAGE (Section 2.2.6.4), the proteins were transferred to a nitrocellulose membrane. The Hybond CTM nitrocellulose membrane (AEC Amersham) and two sheets of filter paper (Whatman) were cut to the same size as the unstained SDS-PAGE gel. After the membrane and gel had been soaked in transfer buffer (48 mM Tris-HCl; 39 mM glycine; pH=9.0) for 30 min, the proteins were electroblotted onto the membrane by using the PR 800 hybridization chamber from Hoefer. The gel-membrane sandwich was totally immersed in transfer buffer and transfer was for 2 h at 24 V and 100 mA.

After transfer, the membrane was washed 3 times for 5 min with 1 × PBS and non-specific binding was blocked by soaking the membrane in blocking solution (1 % fat-free milk powder in 1 × PBS). Primary antibody (anti-BTV NS2 (Uitenweerde *et al.*, 1995)), diluted 1:100 in fresh blocking solution, was added and the membrane was incubated at room temperature for 2 h with agitation. After washing the membrane 3 times for 5 min

with wash buffer (0.05 % Tween-20 in $1 \times$ PBS) to remove unbound antibodies, a secondary antibody, diluted 1:1000, was added (horseradish peroxidase conjugated swine anti-rabbit IgG; Sigma) and incubated at room temperature for 1 h with agitation. The membrane was washed 3 times with wash buffer and then once with $1 \times$ PBS before incubation of the membrane in enzyme substrate (60 mg 4-chloro-1-naphthol in 20 ml ice-cold methanol; 60 μ l of H_2O_2 in 100 ml of $1 \times$ PBS) until the bands became visible.

2.2.7 Nitrocellulose RNA-protein binding assay

Cytoplasmic extracts prepared from recombinant baculovirus-infected and mock-infected *Sf* cells, and *in vitro* synthesized radiolabelled RNA were subjected to nitrocellulose-binding assays according to the procedure described by Huisman *et al.* (1987), with the following modifications. Radioactively labelled RNA transcripts were mixed with fixed amounts of the respective S10 fractions (Section 2.2.6.3). After agitation for 30 min at 4 °C, the mixtures were diluted to 1 ml with 0.01 M STE (10 mM NaCl; 10 mM Tris-HCl; 1 mM EDTA; pH=7.4) and filtered through a Hybond CTM nitrocellulose membrane (Amersham) using a SlotBlot apparatus (Biorad). The membranes were washed seven times with buffers of increasing ionic strength (0.15 M NaCl to 0.5 M NaCl), dried and the radioactivity counted in a scintillation counter. The results are presented graphically as the percentage of radioactively labelled RNA bound to the BTV NS2 protein in relation to the total amount of radiolabelled RNA added to the reaction. The binding assay is presented diagrammatically in Fig. 2.3.

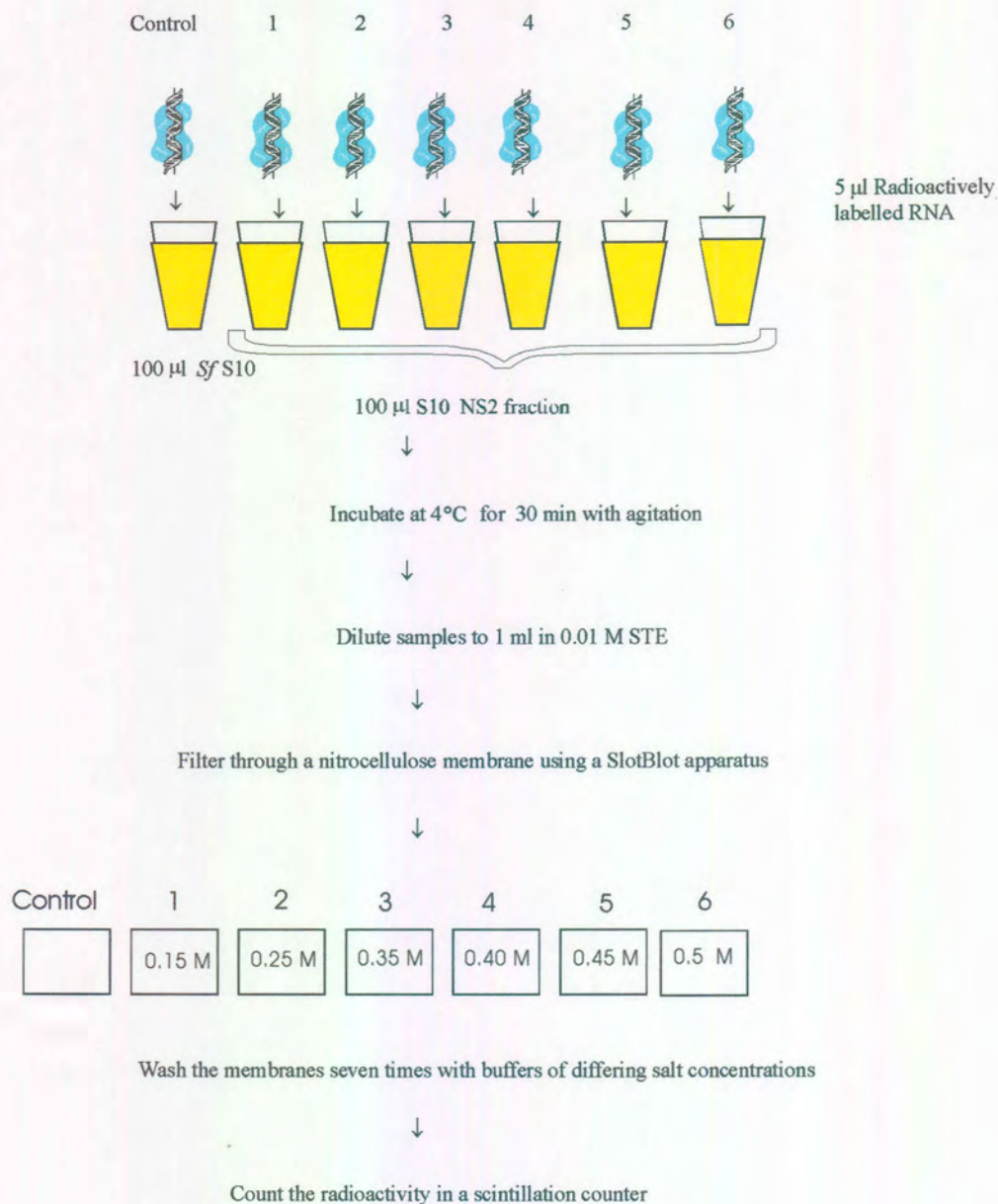


Fig. 2.3: Diagram of the experimental procedures used in a nitrocellulose-binding assay to investigate the importance of the inverted repeat sequences in RNA-protein binding.

2.3 RESULTS

2.3.1 Construction of mutant copies of the NS2-encoding gene

By making use of different combinations of primers (Fig. 2.1) and the amplification conditions described in Section 2.2.1.1, three mutant NS2 genes were constructed of which the 5' and/or 3' terminal sequences were deleted. Unique *Bam* HI restriction sites were included at the 5' end of each primer to simplify subsequent cloning and screening procedures. A three base pair-clamping sequence was added to the 5' end of each primer to allow greater stability of the restriction enzyme site during restriction enzyme digestion. Following PCR amplification, using a full-length clone of the BTV NS2 gene as a template, single discrete DNA bands were synthesized. No amplification was observed in the control reaction where no template DNA was added, indicating that amplification resulted only from the addition of a BTV NS2 gene-specific DNA template. Two PCR reaction mixtures for each of the respective amplicons were pooled and the DNA precipitated by ethanol precipitation. The respective PCR products were analyzed on a 1 % agarose gel (Fig. 2.4) and a DNA molecular size marker was included on the gel to determine the size of the amplified products. The size of the amplified products corresponded to approximately 1.1 kbp, which is in agreement with the expected size of the NS2 gene.

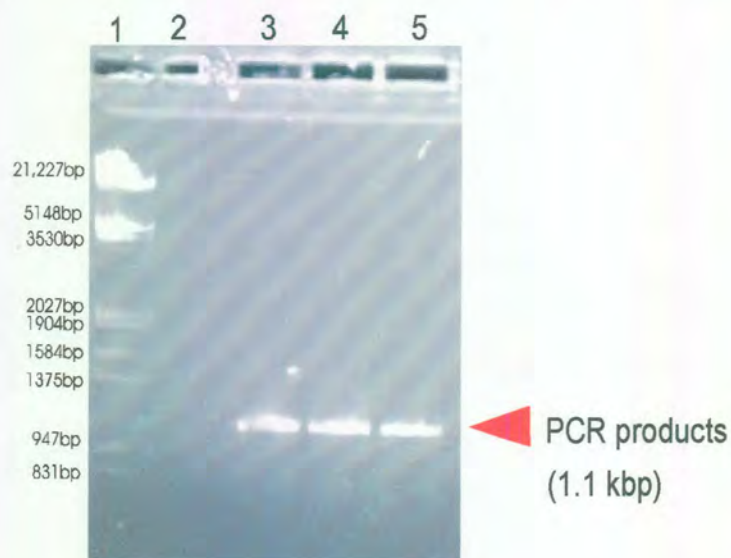


Fig. 2.4: Agarose gel electrophoresis of the amplicons obtained using the primers as described in Fig. 2.1. The sizes of the marker Lambda DNA digested with *Eco* RI and *Hind* III are indicated to the left of the figure (lane 1). Lane 2, control reaction lacking template DNA. Amplicons of the expected size are indicated in lane 3 (i5'BTVNS2), lane 4 (i3'BTVNS2) and lane 5 (i5'3'BTVNS2).

The amplicons were digested with *Bam* HI and cloned into the pBS vector. The restriction enzyme digested vector DNA and PCR products were gel-purified using the glass milk and glasswool methods, respectively, and ligated in a 1:3 molar ratio of vector to insert. Following transformation of competent *E. coli* cells (1×10^7 cells/ μ g DNA), white colonies with a Gal⁻ phenotype were picked from the LB agar plates and grown overnight whereafter plasmid DNA was extracted. A blue, nonrecombinant transformant was included as a size marker (Fig. 2.5).



Fig. 2.5: Agarose gel electrophoretic analysis of plasmid DNA extracted from putative recombinant transformants. Lane 1, parental pBS plasmid (blue colony); lanes 2-8, putative i5'BTVNS2 clones; lanes 9-15, putative i3'BTVNS2 clones and lanes 16-22, putative i5'3'BTVNS2 clones.

Plasmid DNA migrating slower than the parental pBS plasmid DNA (Lane 1, Fig. 2.5) were selected for further characterization by restriction enzyme digestion (Lanes 2,3,4,9,10,12,15,16,19,22, Fig. 2.5). Clones containing the 1.1 kbp insert were subsequently subjected to further restriction enzyme analyses to determine the orientation of the cloned insert DNA.



Fig. 2.6: Agarose gel electrophoretic analysis of the recombinant transcription vector after *Bam* HI restriction enzyme digestion. Lane 2, linearized parental pBS plasmid; lanes 3 and 4, uncut and restriction enzyme digested i5'3'BTVNS2 plasmid DNA, respectively; lanes 5 and 6, uncut and restriction enzyme digested i5'BTVNS2 plasmid DNA, respectively; lanes 7 and 8, uncut and restriction enzyme digested i3'BTVNS2 plasmid DNA, respectively. The sizes of the marker *Lamda* DNA digested with *Eco* RI and *Hind* III (Promega) are indicated to the left of the figure (lane 1).

The assumption has been made that BTV viral mRNA are encapsidated with the minus strands subsequently made using the packaged positive strands as templates. It is important that the insert DNA was cloned under control of the T7 RNA polymerase promoter to enable synthesis of only sense transcripts (mRNA) during *in vitro* transcription (Fig. 2.7). The results after restriction enzyme digestion with *Sma* I indicated that a DNA fragment of approximately 620 bp was excised which is in agreement with the expected size if the insert DNA was cloned under control of the T7 RNA polymerase promoter (Fig. 2.8).

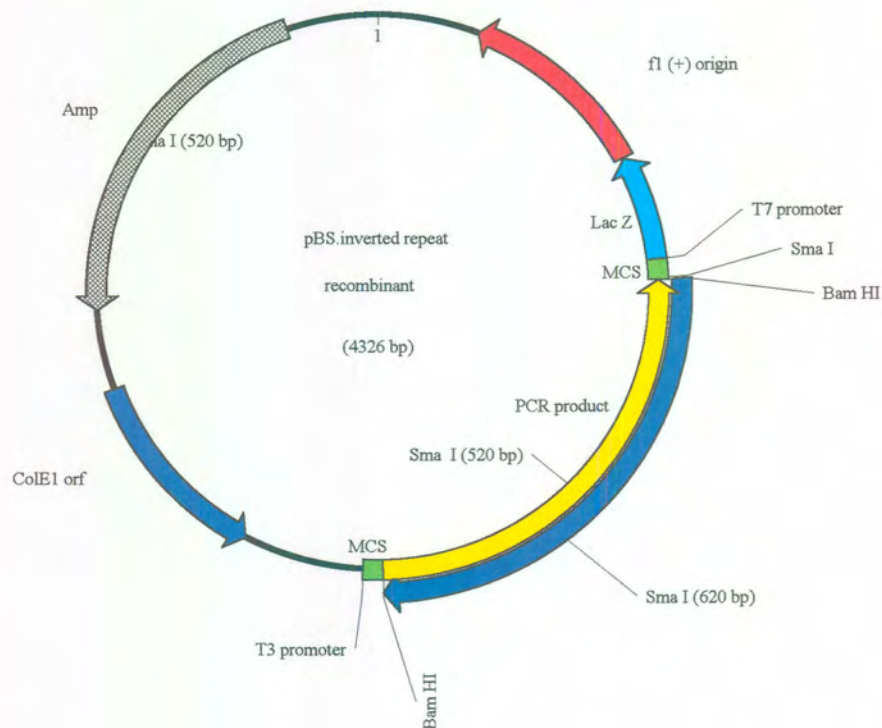


Fig. 2.7: Plasmid map indicating the possible orientations of the cloned insert DNA in the pBS transcription vector. If the PCR products were successfully cloned under control of the T7 RNA polymerase promoter (gene indicated in blue), a DNA fragment of approximately 620 bp would be excised. However, if the cloned insert DNA was cloned in the wrong orientation (gene indicated in yellow), a DNA fragment of only 520 bp would be excised.

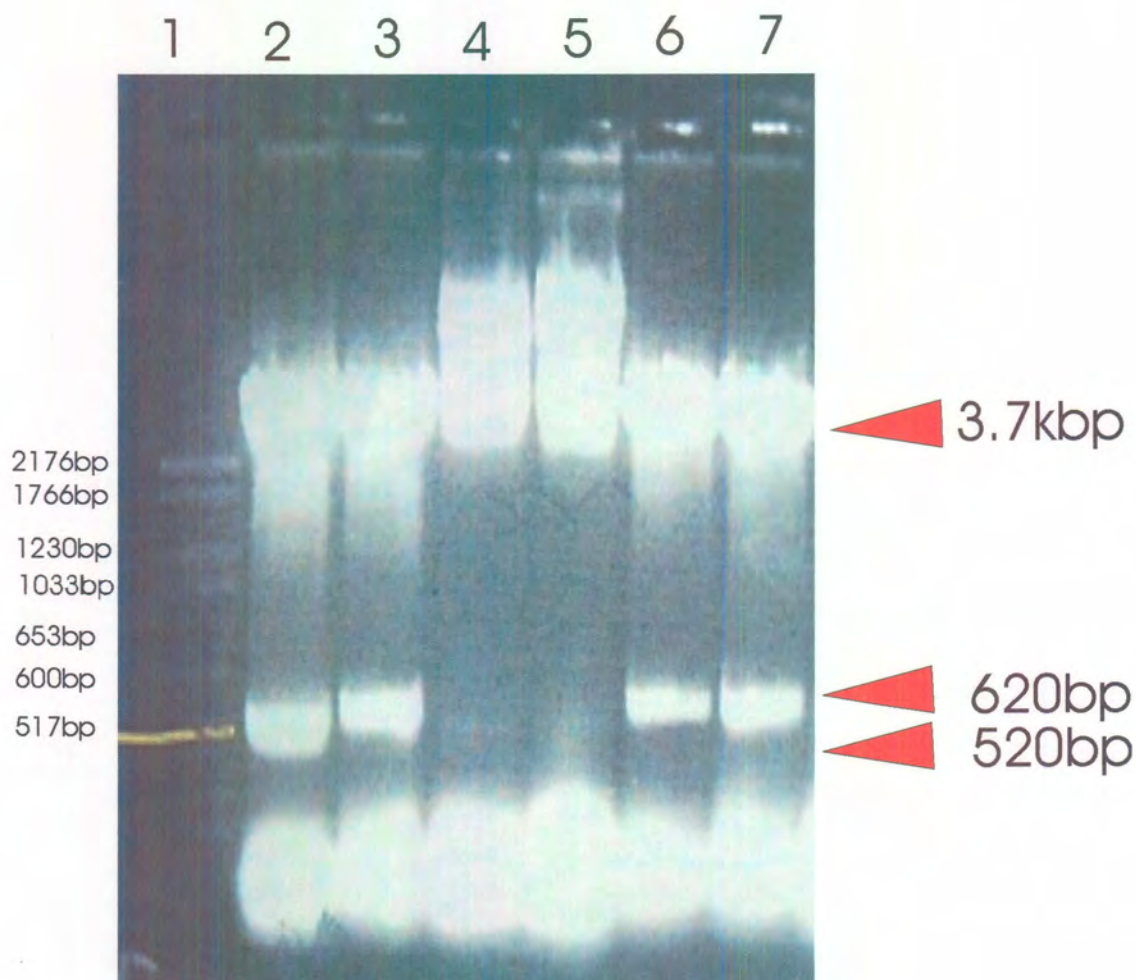


Fig. 2.8: Agarose gel electrophoretic analysis of the recombinant transcription vector constructs indicating the orientation of the cloned inserts. Lane 2, restriction enzyme digested recombinant plasmid DNA (i5'3'BTVNS2) with insert DNA in the incorrect orientation; lane 3, restriction enzyme digested i5'3'BTVNS2 plasmid with insert in the correct orientation; lanes 4 and 5, uncut i5'3'BTVNS2 and i5'BTVNS2 plasmids respectively; lane 6, restriction enzyme digested i5'BTVNS2 plasmid; lane 7, restriction enzyme digested i3'BTVNS2 plasmid. The band sizes of 620 bp (correct orientation) in lane 3, 6 and 7 and band size of 520 bp (incorrect orientation) in lane 2 are indicated to the right of the figure. The sizes of the marker IV (Roche diagnostics) are indicated to the left of the figure (lane 1).

To verify the integrity of the introduced deletions in the respective mutant NS2 genes, automated sequencing was performed. Primers that specifically anneal to the pBS vector

sequences flanking the cloned insert DNA were used in the sequencing reactions. The samples were analysed on an ABI 377 sequencer and the results confirmed that the desired deletions were successfully generated at the 5' and 3' ends of the cloned DNA of the recombinant plasmids (i5'3'BTVNS2, i3'BTVNS2 and i5'BTVNS2).

2.3.2 *In vitro* transcription

In order to obtain RNA transcripts of the respective mutant genes, the recombinant pBS transcription vectors were linearized with *Xba* I that cuts directly after the 3' end of the cloned insert DNA in the multiple cloning site of the vector. Following glassmilk-purification of the linearized restriction fragments, radioactively labelled *in vitro* run-off transcripts were prepared using T7 RNA polymerase. After transcription, the RNA was precipitated to remove unincorporated ribonucleotides and then analyzed on a 1 % agarose gel followed by ethidium bromide staining. The RNA bands were excised from the gel and counted in a scintillation counter to determine the degree of RNA-labelling (Table 2.2).

2.3.3 Expression of the BTV NS2 protein in the baculovirus expression system

The BTV NS2 protein used for investigations regarding RNA-NS2 protein binding was expressed in a eukaryotic expression system. A recombinant baculovirus containing the full-length NS2 gene under control of the strong polyhedrin promoter has previously been generated (Uitenweerde *et al.*, 1995). To express the NS2 protein, *Sf* cells were seeded in tissue culture flasks and infected with the baculovirus recombinant at a MOI of 5 pfu/cell. After 72 h, the baculovirus-infected cells as well as control mock-infected cells were harvested and cytoplasmic extracts were prepared. Samples were analyzed on a SDS-PAGE gel and stained with Coomassie blue to visualize the proteins. The results (Fig. 2.9) indicated that a protein of 42 kDa was expressed in cells infected with the recombinant baculovirus. This corresponded to the size of the expected BTV NS2 protein. This protein band was not present in a cytoplasmic extract prepared from mock-infected *Sf* cells.

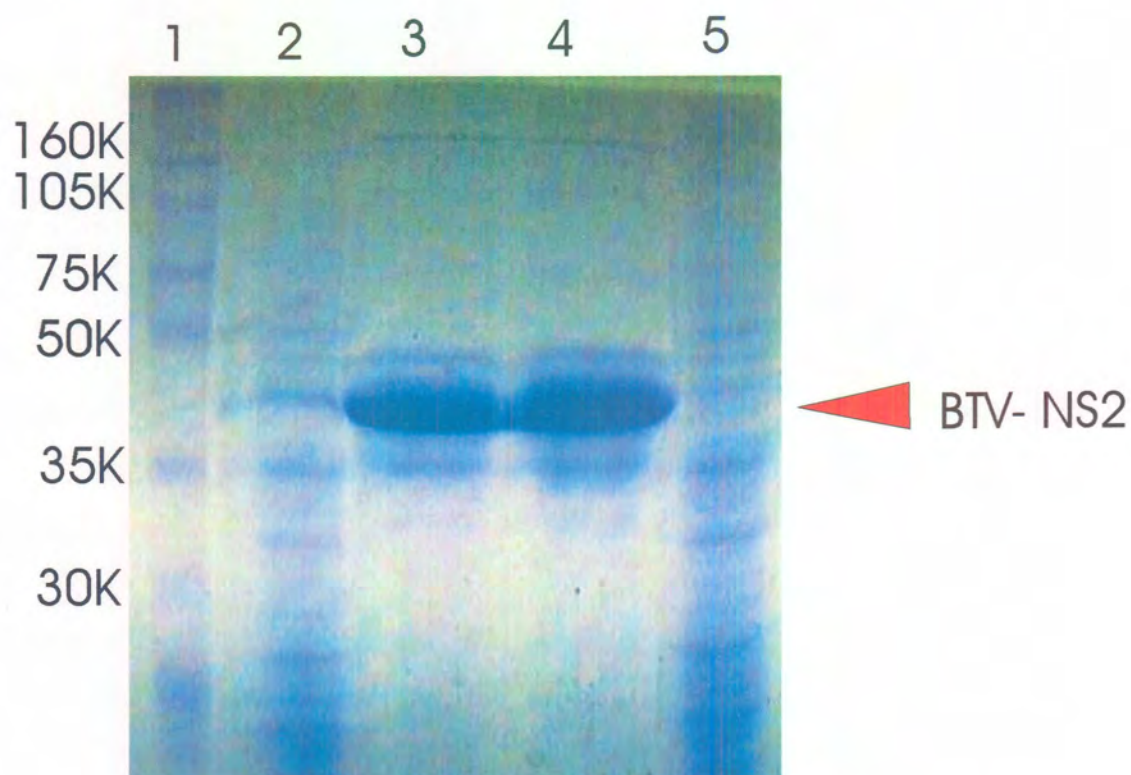


Fig. 2.9: SDS-PAGE analysis of cellular extracts prepared from recombinant baculovirus-infected cells expressing BTV NS2 and mock-infected *Sf* cells. Molecular weights of the marker in kilodalton (lane 1) (AEC Amersham) are indicated to the left of the figure. Lanes 2 and 5, mock-infected *Sf* cells; lanes 3 and 4, recombinant baculovirus-infected cells expressing the BTV NS2 protein. The position of NS2 is indicated to the right of the figure.

An unstained copy of the gel was subjected to Western blot analysis to verify that the protein was indeed the NS2 protein (Fig. 2.10). By using a BTV NS2 antiserum, the results obtained indicated that a single band of 42 kDa reacted with the antiserum, thereby verifying that the uniquely expressed protein was indeed the BTV NS2 protein. No cross-reaction of insect cell proteins with the BTV NS2 antiserum was observed.



Fig. 2.10: Western blot analysis verifying the authenticity of the baculovirus-expressed BTV NS2 proteins. Molecular weights of the protein marker (AEC Amersham), in kilodalton, are indicated to the right of the figure (lane 5). Mock-infected cells were included as controls (lanes 1 and 4). Lanes 2 and 3 contain recombinant baculovirus-expressed NS2.

2.3.4 Nitrocellulose RNA-protein binding assay

Since the BTV NS2 protein appears to bind non-specifically to ssRNA species, it was investigated whether the inverted repeat sequences on the BTV mRNA segments may be required for specificity in the RNA-protein interaction. Therefore, an important objective of this study was to select a RNA-protein binding assay that allows accurate quantitative measurements of the degree of binding. By making use of a nitrocellulose RNA-protein binding assay, it was attempted to measure not only whether binding occurred or not, but also to quantify the degree of protein-RNA binding.

Fixed amounts of the NS2 protein and radiolabelled RNA were allowed to interact with each other. The reaction mixture was filtered through a nitrocellulose membrane to allow

the RNA-NS2 complexes to bind to the membrane. The membrane was washed with a stepwise increase in salt concentration. This stepwise increase in the salt concentration was used to measure changes in the degree of binding. At higher salt concentrations, only those RNA-protein complexes which interact strongly would remain complexed. After drying the membranes, the remaining radioactivity was counted in a scintillation counter (Table 2.2) and the results are indicated graphically in Fig. 2.11. Control reactions performed by using mock-infected *Sf* cells and the full-length BTV segment 8 transcript indicated that none of the *Sf* proteins bound to the RNA transcripts (data not shown). Therefore, the scintillation counts represent binding of the respective transcripts to the BTV NS2 protein only.

Table 2.2: Scintillation counts (cpm) of the labelled transcripts after *in vitro* transcription reactions and of nitrocellulose membranes after binding experiments at different salt concentrations

	wtBTVNS2	Δ mBTVNS2	i5'3'BTVNS2	i5'BTVNS2	i3'BTVNS2
*Labelled transcripts	371 980	368 987	403 767	417 380	370 447
0.15 M NaCl	137 560	123 547	54 653	5 625	9 117
0.25 M NaCl	130 620	114 437	34 872	4 592	6 480
0.35 M NaCl	106 193	96 115	31 313	3 777	5 700
0.4 M NaCl	104 899	95 066	21 462	3 990	5 020
0.45 M NaCl	92 340	88 526	26 665	2 870	5 278
0.5 M NaCl	69 900	64 947	25 462	2 947	2 163

* Scintillation counts of 5 μ l of labelled RNA transcripts after *in vitro* transcription and removal of unincorporated ribonucleotides. An equal amount of radiolabelled RNA (370 000 cpm) was added to each binding reaction.

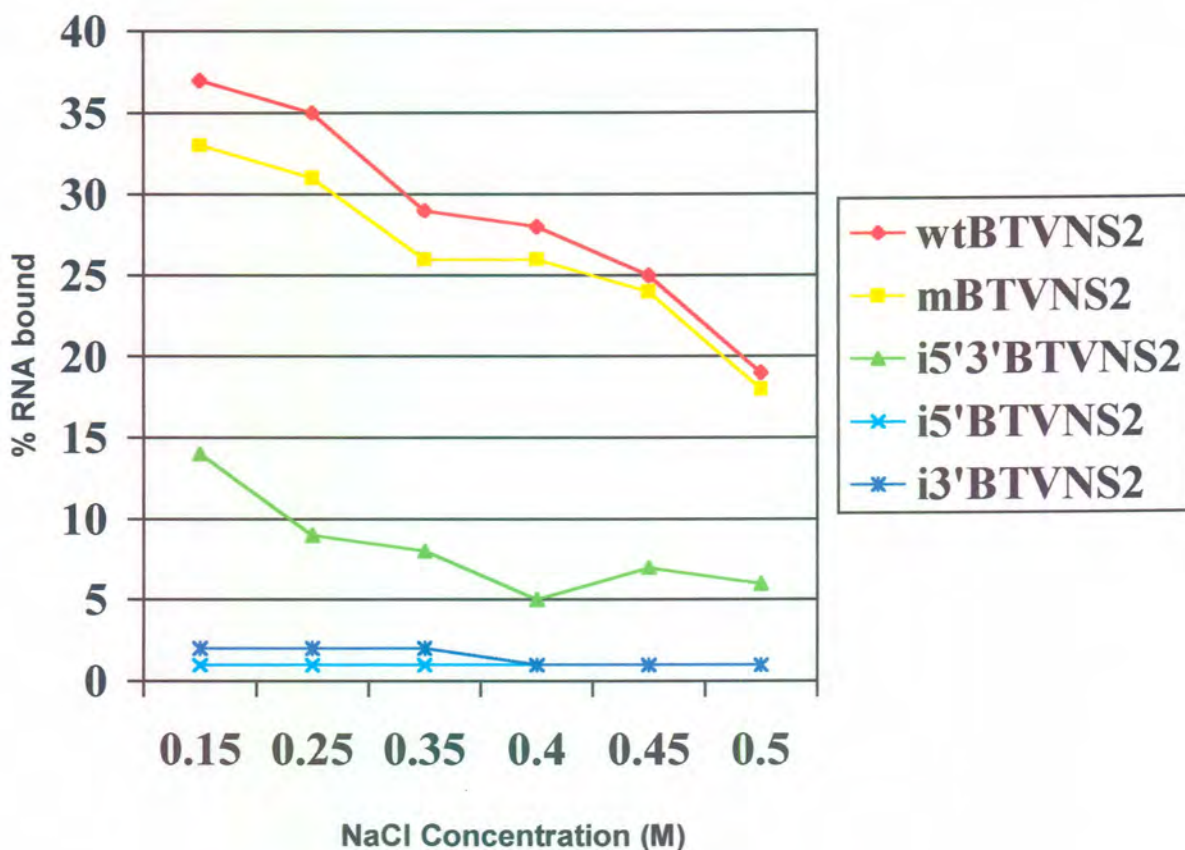


Fig. 2.11: Analysis of the ssRNA-binding ability of baculovirus-expressed BTV NS2 to labelled transcripts using a nitrocellulose-binding assay. The different RNA transcripts used in this assay are indicated to the right of the figure.

The results (Fig. 2.11) indicated that the transcripts lacking the 5' and/or 3' inverted repeat sequences, all displayed weaker binding to the NS2 protein when compared to the full-length BTV NS2 transcript (wtBTVNS2) and the BTV NS2 transcript in which the hexanucleotide sequences were removed (Δ mBTVNS2). The most drastic decrease in RNA-NS2 binding was observed in the deletion mutants lacking either the 5' or 3' inverted repeat sequence. Only 1 % of the respective RNA transcripts were bound at 0.15 M NaCl compared to 37 % of the full-length transcript. For the deletion mutant lacking the 5' and 3' inverted repeat sequences (i5'3'BTVNS2), 14 % of the mutant transcript was bound to NS2 at a salt concentration of 0.15 M NaCl and this steadily

decreased to 6 % at 0.5 M NaCl. In contrast, 17 % of the full-length transcript still remained bound to the NS2 protein at this salt concentration. As expected, mutant transcripts lacking the terminal hexanucleotide sequences displayed a binding affinity similar to the full-length transcripts over the salt concentrations tested. These latter results are in agreement with those reported by Theron and Nel (1997).

2.4 DISCUSSION

The mechanism whereby orbiviruses can selectively recognize and package the ten different RNA genome segments is still unknown. Since the single-stranded RNA binding protein NS2 may be involved in this process, our approach towards understanding specificity in such interactions relies on identifying those elements on the viral RNA that may be involved in NS2 binding. All ten BTV RNA segments contain conserved hexanucleotide sequences at their 5' and 3' ends. Initially, it was thought that these sequences may play a role in RNA-binding but subsequent investigations indicated that they are not directly involved in these interactions (Theron and Nel, 1997). A second characteristic of the orbiviral RNA is the presence of segment-specific inverted repeat sequences, located adjacent to the conserved hexanucleotide sequences. These sequences differ in nucleotide sequence composition and length for each of the RNA segments.

In this part of the study, three different deletion mutant NS2 genes, where either one or both of the inverted repeat sequences were deleted, were constructed. A nitrocellulose-binding assay was then used to investigate the binding interaction between the BTV NS2 protein and the different mutant RNAs. It was subsequently shown that the binding of BTV RNA transcripts to the BTV NS2 protein is influenced by the absence of the inverted repeat sequences on the mRNA. Deletion mutants where either the 3' or 5' inverted repeat sequence were removed displayed weaker binding to NS2 compared to the mutant mRNA lacking both the 5' and 3' inverted repeat sequences. It may be that the weaker binding of these mutant transcripts to NS2, compared to the full-length transcript, is the result of an alteration in the secondary structure of the mRNA, rendering binding sites unavailable to the protein.

Although inverted repeat sequences have been identified in the genomes of various different viruses, their role in the replication cycle of the viruses has not yet been

elucidated. However, in the case of wound tumor virus (WTV) it has been reported that a protein present in extracts prepared from WTV-infected *A. constricta* leafhopper cells binds RNA transcripts in a structure-dependant manner (Dall *et al.*, 1990). RNA-protein binding was investigated using a gel retardation assay and full-length WTV S8 transcripts as well as a series of analogues that contained modified terminal or internal sequences. Transcripts in which the terminal inverted repeats were removed, led to a 10-fold reduction in binding compared to full-length WTV S8 transcripts. A mutant transcript with intact inverted repeat sequences but lacking 466 nucleotides from the internal portion of the gene bound as well as full-length WTV S8 transcripts to the protein. In contrast, a transcript containing only the 64 nucleotides from the 5'-terminus and 32 nucleotides of the 3'-terminus, bound 15-fold less efficient to the protein than the full-length transcript. This indicated that the inverted repeat sequences by themselves are not sufficient for optimal complex formation and that other internal secondary structures may be involved in binding which may be located near the ends of the RNA transcripts. Alternatively, RNA-protein interactions may also occur through binding of the protein to the RNA, irrespective of its secondary structure. By making use of UV cross-linking and gel retardation assays, Poncet *et al.* (1994) reported that binding of NSP3 to RNA, the rotavirus analogue of BTV NS2, occurs in a sequence-specific manner. NSP3 binds to a consensus sequence present at the 3' end of all group A rotavirus mRNA. Only four of these bases (GACC) are required for optimal RNA-protein binding.

The results obtained in this part of the investigation regarding RNA-NS2 binding appear to be in agreement with those described by Dall *et al.* (1990) and indicate that optimal RNA-NS2 interaction may require the presence of the inverted repeat sequences in the RNA transcript. This may be due to the ability of full-length S8 RNA segments to form higher order secondary structures through base-pairing at the inverted repeat sequences which might be the preferred substrates for NS2 binding. In Chapter 4, the secondary structure of the mutant RNA transcripts, in comparison to the full-length BTV mRNA transcripts, are indicated and possible reasons for the observed decline in RNA-protein binding, is further discussed.

CHAPTER 3:
COMPARATIVE BINDING ANALYSIS OF BTV, EHDV AND AHSV SEGMENT 8 RNA
TO THE BTV NS2 PROTEIN USING AN IASYS BIOSENSOR

3.1 INTRODUCTION

One of the aims of this investigation was to investigate specificity in the RNA-NS2 interaction. In the previous chapter, RNA-protein binding was investigated by means of a nitrocellulose-binding assay to determine the importance of inverted repeat sequences on the mRNA for interaction with NS2. In addition, the nitrocellulose-binding assay was expensive and generates excessive radioactive waste. In this chapter, the applicability of an IAsys biosensor to investigate specificity in RNA-NS2 binding was investigated by attempting to quantify the binding of NS2 to mRNA segments obtained from different orbiviruses. If the actual role of the NS2 protein in the BTV replication cycle is to select the 10 ssRNA segments for replication, specificity in the NS2–mRNA interaction towards BTV mRNA segments should be demonstrated.

During the course of this investigation, several different approaches were used to investigate specificity in RNA-NS2 interaction. The details of these investigations are not presented here, but will be briefly summarized. Most of these initial approaches relied on UV cross-linking analysis as a means whereby proteins, which are in close, direct contact with RNA, can be covalently linked to the RNA (Leipoldt and Munro, 1988). Initial attempts relied on mixing S10 extracts prepared from BTV-infected BHK cells with unlabelled ssRNA transcripts to allow binding to occur whereafter the complexes were immobilized on a nitrocellulose membrane and treated with RNase to remove all unbound RNA. The membrane was then probed with radioactively labelled homologous DNA probes. Interpretation of results was difficult due to radioactivity observed in binding experiments with fractions prepared from uninfected BHK cells, indicating non-specific binding of the labelled RNA to the BHK cellular proteins. Detection of radioactivity may also be due to inefficient RNase digestion of unbound RNA, leading to detection of remaining RNA with the radioactively labelled DNA probe. To minimize non-specific binding, the samples were immunoprecipitated with BTV NS2-specific antiserum prior to immobilization on the membrane. Once again, background was observed since the labelled RNA bound to the protein A conjugate used for immunoprecipitation. A variation of the above approach relied on mixing a S10 fraction containing the BTV NS2 protein with radiolabelled RNA transcripts and then subjecting the sample to UV cross-linking prior to SDS-PAGE and autoradiography of the immunoprecipitated samples. Using this approach, it was relatively easy to determine whether binding occurred or not, but the binding could not be quantified. As an alternative approach, a North-western blot was

also used. The protein extracts were first electrophoresed on a SDS-PAGE gel, transferred onto a membrane and then probed with radiolabelled RNA transcripts. No binding was observed. This may have been due to an alteration of the conformation of the protein, which may be important for RNA-binding.

As it was important to accurately quantify the interaction of the NS2 protein to different ssRNA transcripts, the use of an optical biosensor as a means whereby binding efficiency could be quantified was subsequently investigated. The biosensor relies on the detection of changes in the refractive index as a ligate (RNA) binds to a ligand (protein). One of the binding partners (in this study, the protein) must be immobilized on the sensor surface and during this immobilization process the binding activity of the ligand should be retained. The method described by Rubio *et al.* (1997) for investigating protein-protein interactions using a GST fusion protein was of particular importance for this study, because the BTV NS2 protein was expressed as a GST-fusion protein. The authors indicated that when the GST fusion protein was directly bound to a carboxymethyl dextran matrix (CMD), it resulted in a complete loss of its binding activity. However, the binding ability of the GST protein was retained when immobilized by a 6-carbon spacer.

In this part of the investigation, three different transcription vectors were constructed to generate full-length segment 8 mRNA transcripts of BTV, EHDV and AHSV, and the NS2 protein was expressed in a prokaryotic system as a GST fusion protein. The binding of the NS2 protein to these different mRNA species was then investigated using the IAsys biosensor.

3.2 MATERIALS AND METHODS

3.2.1 Construction of recombinant transcription vectors containing different orbivirus segment 8 genes

3.2.1.1 Large-scale plasmid DNA isolation

The AHSV segment 8 gene has previously been cloned into the *Bam* HI site of the pUC 18 plasmid (M. Uitenweerde, Department of Genetics, University of Pretoria). The EHDV segment 8 gene has also previously been cloned into the *Bam* HI site of pBS under control of the T3 promoter (J. Theron, Department of Microbiology and Plant Pathology, University of Pretoria). These plasmids were transformed into competent *E. coli* JM109

cells and isolated as previously described (Sections 2.2.2.1 and 2.2.2.2). The plasmid DNA was incubated with 1 μ l RNase A (10 mg/ml) (Roche) at 37 °C for 1 h to remove contaminating RNA that may interfere with subsequent restriction enzyme digestions.

3.2.1.2 Restriction enzyme digestions

Plasmids pBS (Stratagene), pBS.EHDVS8 and pUC.AHSVS8 were digested with *Bam* HI (Promega). The appropriate salt concentration was obtained by using the 10 $\times$ buffer B (6 mM Tris-HCl (pH=7.5); 6 mM MgCl₂; 50 mM NaCl; 1 mM DTT) supplied by the manufacturer. The different plasmids (1 μ g) were restriction enzyme digested with 1 U of *Bam* HI enzyme in a final reaction volume of 15 μ l and the reactions were incubated at 37 °C for 1.5 h. The enzymes were then inactivated at 65 °C for 15 min. After restriction enzyme digestion, the DNA fragments were analyzed by agarose gel electrophoresis (Section 2.1.1.2). Uncut plasmid DNA was co-electrophoresed with the samples to determine whether restriction of the DNA did occur. A 100 bp DNA marker (Promega) was included on the gel to determine the size of the restriction fragments.

3.2.1.3 Purification of insert and vector DNA

The restriction enzyme digested DNA derived from the pBS.EHDVS8, pUC.AHSVS8 and pBS plasmids was purified using the glassmilk method as previously described (Section 2.2.2.5) and aliquots were analyzed on a 1 % agarose gel after purification (Section 2.1.1.2).

3.2.1.4 Dephosphorylation of the vector DNA

Vector DNA (pBS) was dephosphorylated to prevent selfligation of the *Bam* HI-restriction enzyme digested DNA (Sambrook *et al.*, 1989). The reaction mixture consisted of 1 U of calf intestinal phosphatase (1 U/ μ l, Roche), 5 μ l of 10 $\times$ buffer (50 mM Tris-HCl (pH 8.5); 0.1 mM EDTA) and 1 μ g *Bam* HI-digested pBS vector DNA. The mixture was incubated at 37 °C for 30 min in a 50 μ l reaction volume. The phosphatase-treated DNA was purified using the glassmilk method (Section 2.2.2.5) before use in ligation reactions.

3.2.1.5 Ligation

The insert DNA, obtained following digestion of pBS.EHDVS8 and pUC.AHSVS8, were ligated to the vector DNA overnight at 15 °C. The reaction mixture consisted of 1 U T4

DNA ligase (Roche), 1 μ l of a 10 $\times$ ligation buffer (660 mM Tris-HCl; 10 mM DTT; 50 mM $MgCl_2$; 10 mM ATP; pH=7.5), 150 ng linearized pBS vector and 450 ng of the respective DNA fragments. The total volume of the reaction was 10 μ l. To determine the efficiency of the dephosphorylation step, the dephosphorylated pBS vector was selfligated.

3.2.1.6 Preparation of competent cells and transformation

Competent *E. coli* JM109 (Promega) cells were prepared according to a method described by Hanahan *et al.* (1991), which allows long-term storage of the prepared cells without significant loss of competency. Colonies from a freshly streaked LB agar plate were inoculated into SOB-Mg growth medium (2 % Bacto-tryptone; 0.5 % Bacto-yeast extract; 10 mM NaCl; 2.5 mM KCl). After overnight culturing, the cells were gently vortexed and inoculated into a 500 ml Erlenmeyer flask containing 50 ml LB broth. The culture was incubated until an OD of 0.3 at 550 nm was obtained. The cell suspension was chilled on ice for 10 min after which the cells were pelleted by centrifugation at 3000 rpm for 10 min in a Sorvall S10 rotor. The supernatant was removed and the cells were suspended in 1/3 of the volume CCMB 80 (80 mM $CaCl_2 \cdot 2H_2O$; 20 mM $MnCl_2 \cdot 4H_2O$; 10 mM $MgCl_2 \cdot 6H_2O$; 10 mM potassium acetate; 10 % (v/v) glycerol) and incubated on ice for 20 min. The cells were pelleted by centrifugation for 10 min at 3000 rpm in a Sorvall S10 rotor. The supernatant was removed and the cells resuspended in 1/12 of the original volume CCMB 80. Competent cells were aliquoted in 1 ml volumes into chilled polypropylene tubes and frozen in liquid nitrogen. The cells were stored at $-70^\circ C$ until further use.

The competent cells were first tested for contamination by plating the cells, without added exogenous DNA, onto an agar plate containing ampicillin. To determine the transformation efficiency, pUC 18 (Roche) of a known concentration, was transformed. After performing these test transformations, 5 μ l of each ligation mixture was added to 200 μ l of competent cells. The cells were allowed to stand on ice for 30 min and heat-shocked at $42^\circ C$ for 90 s. Cells were incubated on ice for 2 min and 800 μ l of SOC medium (2 % Bacto-tryptone; 0.5 % Bacto-yeast extract; 10 mM NaCl; 2.5 mM KCl; 20 mM $MgCl_2$; 20 mM $MgSO_4$; 20 mM glucose) was added. After incubation with agitation at $37^\circ C$ for 1 h, the cells were plated together with 2 % X-Gal (40 μ l) and 10 μ l 100 mM of IPTG onto SOB agar plates supplemented with ampicillin to allow for blue-white colour selection.

3.2.1.7 Small-scale plasmid DNA isolation

Putative recombinant transformants (white colonies) were selected and grown overnight in 3 ml LB broth supplemented with ampicillin. Small-scale plasmid DNA isolations were performed as described in Section 2.2.2.2 and the contaminating RNA was removed by incubating the plasmid DNA with 1 μ l RNase A (10 mg/ml) for 1 h at 37 °C (Section 2.2.2.2). Purified plasmid DNA was analyzed by agarose gel electrophoresis (Section 2.1.1.2).

3.2.1.8 Restriction enzyme digestion

Putative recombinant plasmids (Section 3.2.1.7) were digested with restriction enzymes to determine if an insert DNA had been cloned and also to determine the orientation of the cloned insert. The pBS.EHDV and pBS.AHSV plasmids were digested with *Bam* HI (Promega) to determine if the insert DNA was present (Section 2.2.1.3). As a control, parental pBS vector DNA was also restriction enzyme digested. To determine if the genes were cloned in the correct transcriptional orientation, pBS.EHDV was cut with *Eco* RI (Promega) and pBS.AHSV was cut with *Hind* III (Promega). One μ g of each plasmid was incubated with 1 U of the appropriate enzyme in a final reaction volume of 15 μ l. The appropriate salt concentration was obtained by using the 10 $\times$ buffer supplied by the manufacturer. The reaction mixtures were incubated at 37 °C for 1 h and the results were analyzed by agarose gel electrophoresis (Section 2.1.1.2). A 100 bp DNA size marker (Promega) was included to identify the sizes of the restriction fragments.

3.2.1.9 Preparation of recombinant plasmids

Large-scale plasmid DNA isolations of confirmed recombinant clones were performed using the method described in Section 2.2.4.3 and then purified using PEG-NaCl purification (Section 2.2.2.3). The plasmid DNA was stored at -20 °C until further use.

3.2.2 Expression of the BTV NS2-GST fusion protein in *Escherichia coli*

Competent *E. coli* JM109 cells were transformed with recombinant (Dr. J. Theron: Department of Microbiology and Plant Pathology, University of Pretoria) and parental pGEX plasmids (Stratagene: Brought from NERC, Oxford, 1991) and plated onto LB agar plates supplemented with ampicillin (Section 3.2.1.6).

3.2.2.1 Small-scale expression of the fusion protein

Individual colonies containing either the recombinant or parental plasmids were inoculated into LB broth supplemented with ampicillin and grown overnight with agitation at 37 °C. The overnight culture was diluted 1:10 in fresh medium and incubated at 37 °C with agitation for 1 h. Freshly prepared IPTG (5 µl of a 100 mM solution) was used to induce NS2 protein expression. The cultures were vortexed and incubated for 3 h with agitation at 37 °C. After harvesting the cells by centrifugation at 7000 rpm in a Hettich Mikro-12 rotor for 10 min, the supernatant was discarded and the pellet suspended in 100 µl 1 × PBS. An equal volume of 2 × PSB was added and the samples were boiled for 5 min at 100 °C. The bacterial lysates were then subjected to SDS-PAGE analysis. The pGEX vector, expressing only the GST protein, was included as a control.

3.2.2.2 Large-scale expression of the fusion protein

After identifying a colony that expresses the GST-NS2 fusion protein (Section 3.2.2.1), large-scale expression of BTV GST-NS2 was done. A single bacterial colony containing either pGEX-NS2 or pGEX was picked from an agar plate and inoculated into 1 ml LB broth containing ampicillin. The culture was grown overnight at 37 °C with agitation and diluted 1: 10 with fresh medium. After incubation for 3 h, protein expression was induced with 50 µl 100 mM IPTG and incubation was continued for a further 6 h prior to lysing the cells.

3.2.2.3 Lysis of the bacterial cells

Bacterial cells from a 10 ml culture were pelleted by centrifugation for 10 min at 7000 rpm in a Sorvall S10 rotor. The pellet was resuspended in 1 ml Mix 1 (20 % sucrose; 100 mM Tris; 10 mM EDTA) and three freeze-thaw cycles were performed by freezing the cells at -70 °C and then thawing them at 60 °C. The suspension was incubated at room temperature for 15 min and then on ice for 30 min with intermittent vortexing. After the addition of 10 µl DTT, 10 µl Tween-20, 10 µl Triton X-100 and 100 µl 10 × PBS, the cells were incubated for 1.5 h on ice and vortexed every 10 min. The cellular debris were removed by centrifugation at 5000 rpm for 15 min in a Hettich Mikro-12 rotor and the supernatant stored at - 70 °C.

3.2.2.4 SDS-polyacrylamide gel electrophoresis

Samples were analyzed using SDS-PAGE as described in Section 2.2.6.4. The protein samples (100 μ l) were mixed with equal volumes of 2 $\times$ PSB, boiled for 5 min and sonicated for 15 min before being loaded onto a SDS-PAGE gel. A discontinuous gel electrophoresis system was used as described in Section 2.2.6.4. Two gels were electrophoresed in duplicate, one of which was stained with Coomassie brilliant blue (Section 2.2.6.4) and the second, unstained gel was used in a Western blot.

3.2.2.5 Western blot analysis

GST-NS2 fusion proteins, expressed by the recombinant pGEX prokaryotic expression vector, were identified using western blot analysis as described in Section 2.2.6.5.

3.2.3 Preparation of *in vitro* RNA transcripts

3.2.3.1 Linearization of plasmid DNA

RNA transcripts were prepared by run-off transcription following linearization of the recombinant transcription vectors (pBS.EHDV, pBS.AHSV and pBS.wtBTVS8) with *Xba* I (Promega) (Section 2.2.5.1).

3.2.3.2 Purification of linearized plasmids

The linearized DNA fragments were purified using phenol-chloroform extraction. The volume of 100 μ l of linearized plasmid was adjusted to 300 μ l with ddH₂O. Equal volumes of phenol and chloroform were added to the reaction and the mixture was vortexed and centrifuged for 5 min at 15 000 rpm in a Hettich Mikro-12 rotor. The upper aqueous phase was transferred to a new Eppendorf tube and extracted twice with an equal volume of chloroform. The upper aqueous phase was transferred to a new tube and 1/10th of the volume 3 M NaOAc (pH=7) and 2 volumes of 100 % ethanol was added. The DNA was precipitated at -20 °C for 1 h and centrifuged for 20 min at 15 000 rpm in a Hettich Mikro-12 rotor. The pellet was washed three times with 70 % ethanol, dried in a vacuum dryer and resuspended in 20 μ l DEPC water.

3.2.3.3 *In vitro* transcription

Unlabelled RNA transcripts were synthesized in 20 μ l reaction mixtures containing 40 mM Tris-HCl (pH 7.5); 6 mM MgCl₂; 10 mM NaCl; 2 mM spermidine; 10 mM dithiothreitol; 2.5 mM ribonucleotides (ATP, GTP, UTP, CTP); 0.5 μ g of linearized template DNA; 20 U

RNasin (Promega) and 20 U T7 RNA polymerase (Promega). The reaction mixtures were incubated at 37 °C for 1.5 h. Template DNA was destroyed by treatment with 10 U RNase-free DNase (Roche) for 15 min at 37 °C. The RNA was precipitated to remove unincorporated nucleotides (Section 2.2.5.4) and the quality of the transcripts was analyzed by agarose gel electrophoresis (Section 2.1.1.2).

3.2.4 RNA-protein binding analysis using a biosensor

For these experiments a dual-well IAsys cuvette (AEC Amersham) was used and prepared according to the method described by Rubio *et al.* (1997). The CM dextran cuvette was inserted into the IAsys biosensor (AEC-Amersham) and equilibrated in 60 µl PBS–Tween (0.05 %) for 10 min. The cuvette's surface was activated by three successive activation cycles using 60 µl of EDC and NHS (1:1 EDC:NHS). After washing three times with 200 µl PBS–Tween (0.05 %), baseline data (1 min) was collected. Binding buffer was added to the cuvette (60 µl of 0.15 M STE buffer (pH =7)) and after collecting baseline data for 1 min, the AHA spacer (60 µl of a 10 mg/ml stock solution) was immobilized, followed by washing three times with 60 µl PBS–Tween to remove unbound spacer. Unreacted NHS–esters were blocked by the addition of 60 µl of 1 M ethanolamine (pH=8.5) followed by washing three times with 60 µl of PBS–Tween (0.05 %). One min of baseline data was then collected. The GSH protein was immobilized on the surface of the prepared cuvette by adding 60 µl of GSH protein (10 mg/ml). After binding for 10 min to the surface, the cuvette was washed and blocked, as described above, whereafter the GST-NS2 protein was immobilized. Ten min of binding was allowed and the cuvette was washed three times with 60 µl of PBS–Tween (0.05 %) after which 1 min of baseline data was collected. Unreacted NHS–esters were blocked, the cuvette washed with PBS–Tween (0.05 %) and 0.15 M STE (pH=7), as described above, while baseline data (1 min) was recorded after each of the washing steps. Finally, RNA transcripts were added and the data recorded.

The amount of protein bound to the matrix was determined, using the relationship that 200 arc seconds immobilization response is equivalent to 1 ng/mm² concentration of protein (<http://www.affinity-sensors.com/>).

3.3 RESULTS

3.3.1 Cloning of full-length AHSV and EHDV NS2-encoding genes into pBS

The plasmids, pBS.EHDVS8 (insert under control of the T3 promoter), pUC.AHSVS8 and pBS were isolated, purified and digested with *Bam* HI as described under Materials and Methods. Following agarose gel electrophoresis of the restriction fragments, appropriate bands were purified using the glassmilk method. To improve cloning efficiency, the restriction enzyme digested pBS vector was subsequently dephosphorylated to minimize selfligation of the vector DNA. The efficiency of the dephosphorylation reaction was determined by selfligation of the linearized dephosphorylated vector DNA. Following transformation, a decrease of 95 % in ligation efficiency was observed for the dephosphorylated vector compared to the phosphorylated vector. Ligation reaction mixtures with a molar ratio of 3:1 of insert to vector were prepared and transformed into the competent *E. coli* JM109 cells (1×10^7 cells/ μ g DNA). Putative recombinants (white colonies) were selected, cultured overnight in LB broth and plasmid DNA extracted. Agarose gel electrophoresis was used to identify recombinant plasmid DNA based on their slower migration through the gel compared to the parental pBS vector.

Putative recombinants were selected and for several clones, DNA bands of approximately 1185 bp (pBS-EHDV) and 1166 bp (pBS-AHSV) were excised which corresponded in size to the full-length EHDV and AHSV NS2 genes, respectively (Fig. 3.1).

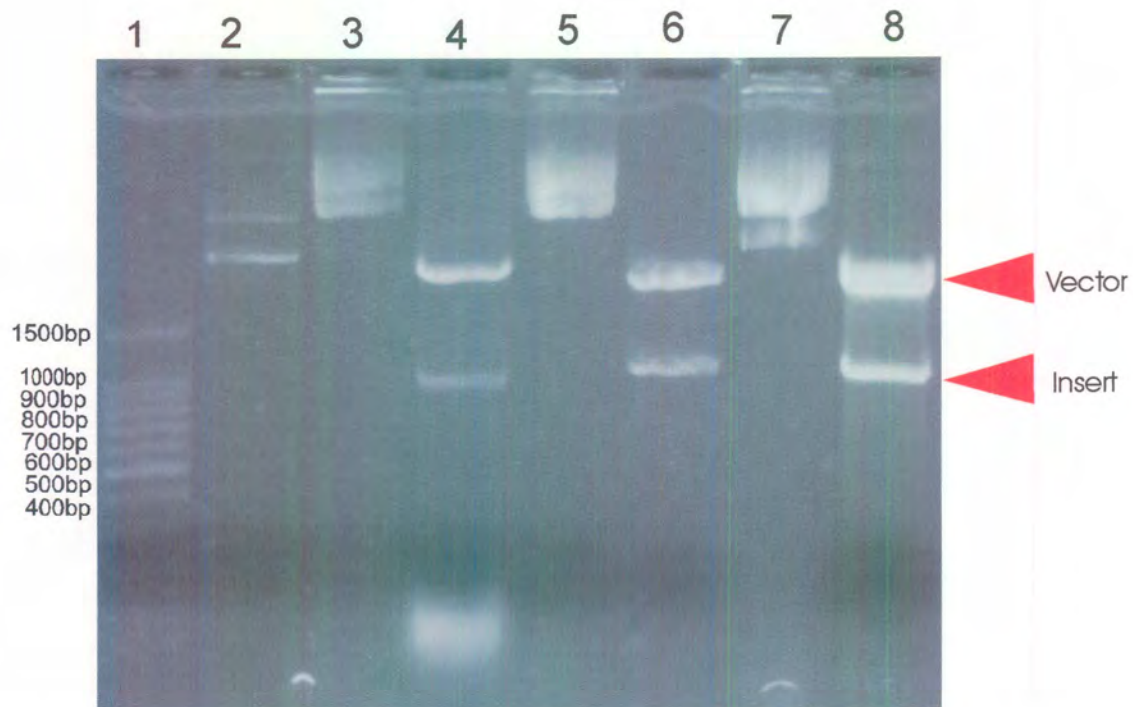


Fig. 3.1: Agarose gel electrophoretic analysis of the putative recombinant pBS transcription vectors following *Bam* HI restriction enzyme digestion. Lane 2, uncut parental pBS plasmid; lane 3, uncut wtBTVNS2 plasmid DNA; lane 4, restriction enzyme digested wtBTVNS2 plasmid indicating the cloned insert DNA of 1124 bp; lane 5, uncut pBS-EHDV plasmid DNA, lane 6, restriction enzyme digested plasmid pBS-EHDV indicating the cloned insert DNA of 1185 bp; lane 7, uncut pBS-AHSV plasmid DNA; lane 8, restriction enzyme digested plasmid pBS-AHSV indicating the cloned insert DNA of 1166 bp. The sizes of the 100 bp Marker (Promega) are indicated to the left of the figure (lane 1).

Recombinant clones containing the cloned EHDV insert DNA were subjected to further restriction enzyme digestion with the *Eco* RI enzyme and asymmetrical digestion patterns were used to determine the orientation of the cloned gene (Fig. 3.2). Recombinant plasmid DNA containing the EHDV insert DNA in the correct transcriptional orientation is depicted in Fig. 3.4 (A).

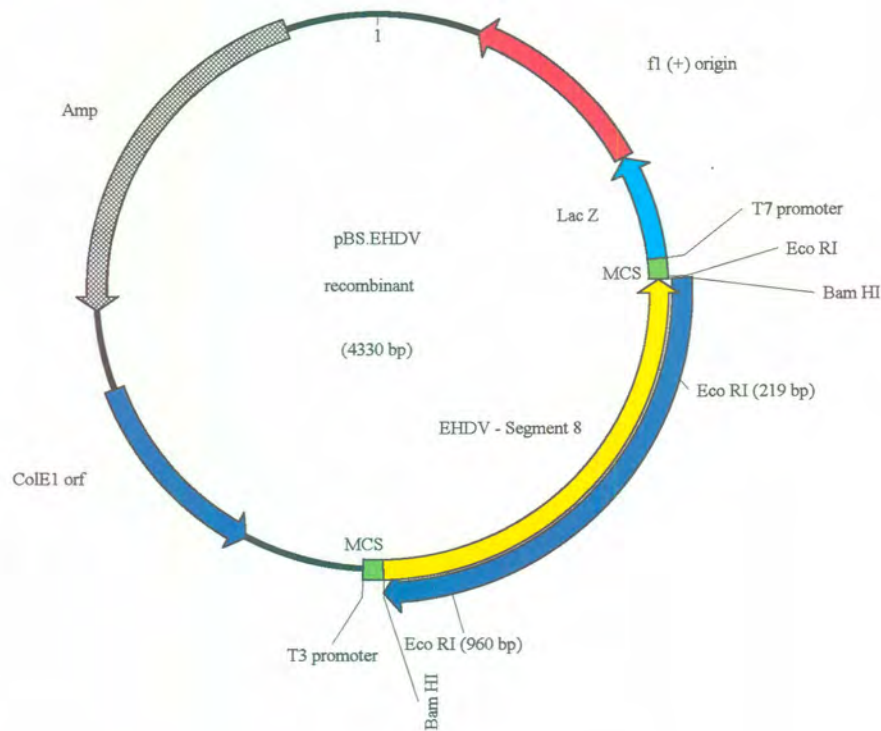


Fig. 3.2: Plasmid map indicating the possible orientations of the cloned EHDV segment 8 DNA in the pBS transcription vector.

The *Hind* III enzyme was used to determine the orientation of the cloned AHSV insert DNA (pBS-AHSV) since it cuts only once in the multiple cloning site of the pBS plasmid and also once in the NS2 gene of AHSV at position 253 (Fig. 3.3). Recombinant plasmid DNA containing the AHSV insert DNA in the correct transcriptional orientation is depicted in Fig. 3.4 (B).

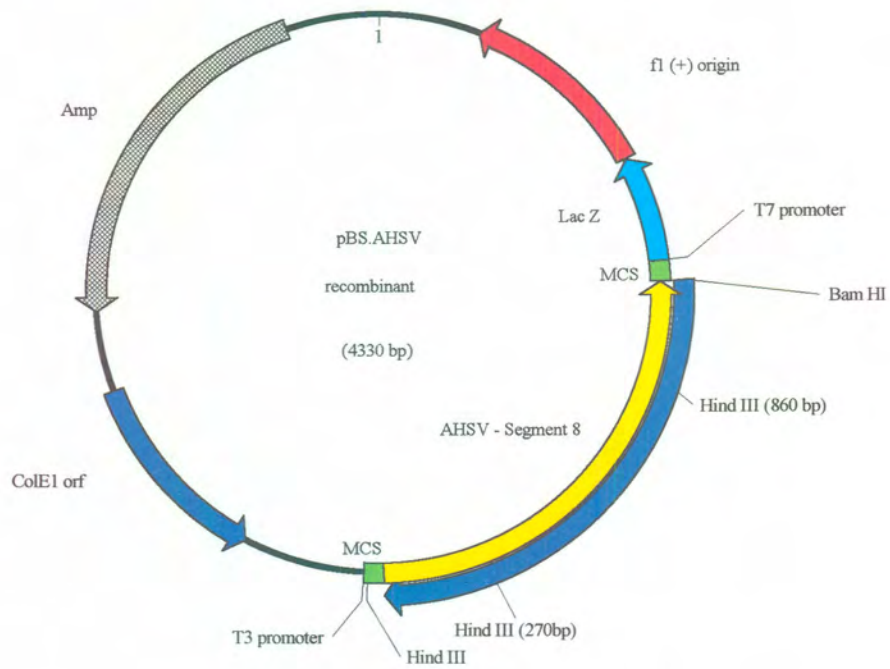


Fig. 3.3: Plasmid map indicating the possible orientations of the cloned AHSV segment 8 DNA in the pBS transcription vector.

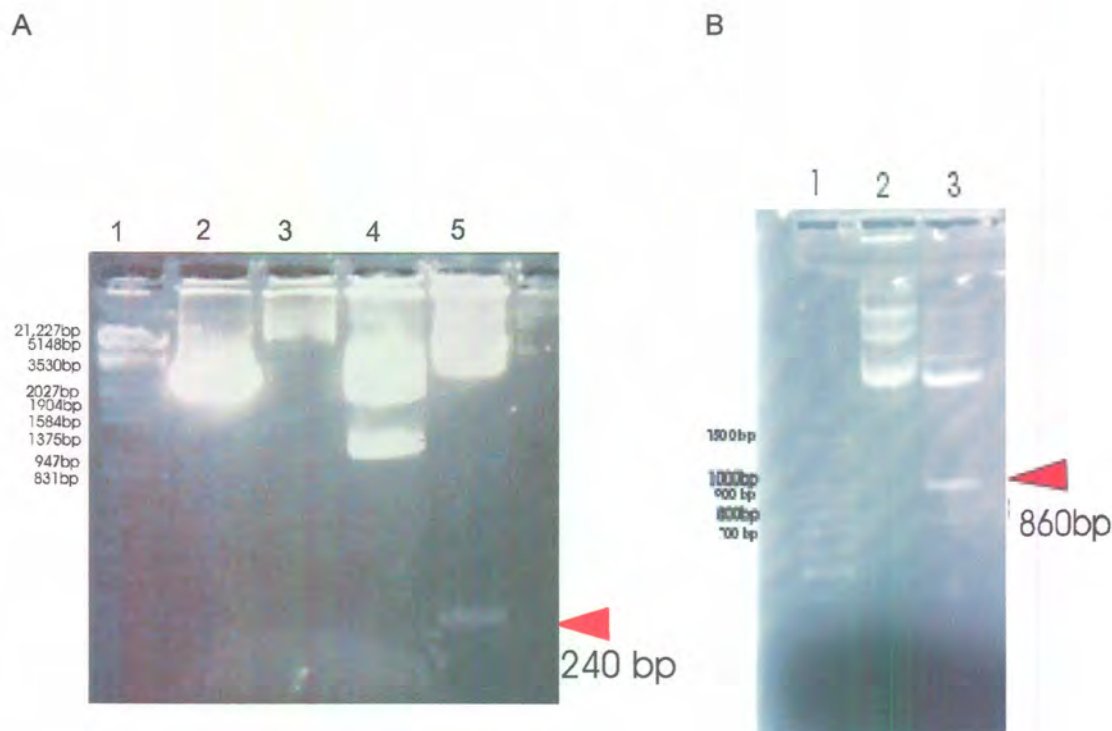


Fig. 3.4 (A): Agarose gel electrophoretic analysis of the EHDV segment 8 recombinant plasmid after *Eco* RI restriction enzyme digestion to verify the orientation of the cloned DNA. Lane 2, uncut pBS plasmid DNA; lane 3, uncut pBS-EHDV plasmid DNA; lane 4, *Bam* HI restriction enzyme digested plasmid pBS-EHDV indicating a 1185 bp band; lane 5, restriction enzyme digested plasmid pBS-EHDV indicating the 240 bp band. The sizes of the marker Lambda DNA digested with *Eco* RI and *Hind* III (Promega) are indicated to the left of the figure (lane 1).

Fig. 3.4 (B): Agarose gel electrophoretic analysis of the AHSV segment 8 recombinant plasmid after *Hind* III restriction enzyme digestion to verify the orientation of the cloned insert DNA. Lane 2, uncut pBS-AHSV plasmid DNA; lane 3, restriction enzyme digested plasmid pBS-AHSV indicating the 860 bp band. The sizes of the 100 bp DNA Marker (Promega) are indicated to the left of the figure.

3.3.2 Expression of the BTV NS2 protein in a prokaryotic expression system

In Chapter 2, the BTV NS2 protein used in the RNA-protein binding experiments was obtained from a baculovirus expression system. In this chapter, a prokaryotic expression system was used to express the BTV NS2 gene. In this pGEX expression system, the

protein is expressed upon induction of transformed cultures with IPTG as a fusion protein containing a GST-tail of 26 kDa. Small-scale expression studies were performed to identify a specific colony that provided adequate levels of GST-NS2 expression whereafter the transformants was cultured in larger (10 ml) volumes to obtain sufficient amounts of protein for subsequent RNA-protein binding assays. Lysates prepared from these cultures were analyzed by SDS-PAGE. The results indicated expression of a protein of 26 kDa (GST protein) in cells containing the parental pGEX vector. In extracts prepared from cells containing the recombinant vector, a 69 kDa protein was expressed (Fig. 3.5).

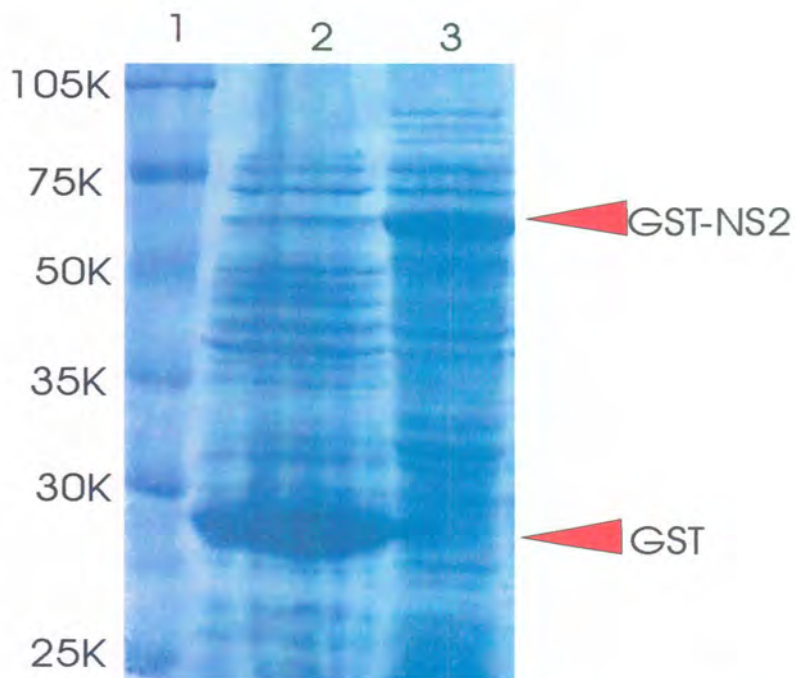


Fig. 3.5: SDS-PAGE analysis of *E. coli* extracts indicating expression of the BTV NS2 protein. Molecular weights of the protein marker, in kilodalton, are indicated (lane 1) (AEC Amersham). Lane 2, *E. coli* cells transformed with the parental pGEX vector resulting in expression of the 26 kDa GST protein. Lane 3, *E. coli* cells transformed with the recombinant pGEX-NS2 vector resulting in expression of the 69 kDa GST-NS2 fusion protein.

To verify that this expressed protein is indeed the BTV NS2 protein, a Western blot was performed using a monospecific BTV NS2 antiserum (Fig. 3.6). A protein band of 69 kDa specifically reacted with the BTV NS2 antiserum, indicating the presence of the bacterial-

expressed NS2 fusion protein. A smaller band also reacted specifically with the antiserum and may represent a degraded derivative of the GST-NS2 protein, as *E. coli* proteases are known to degrade heterologously expressed proteins.

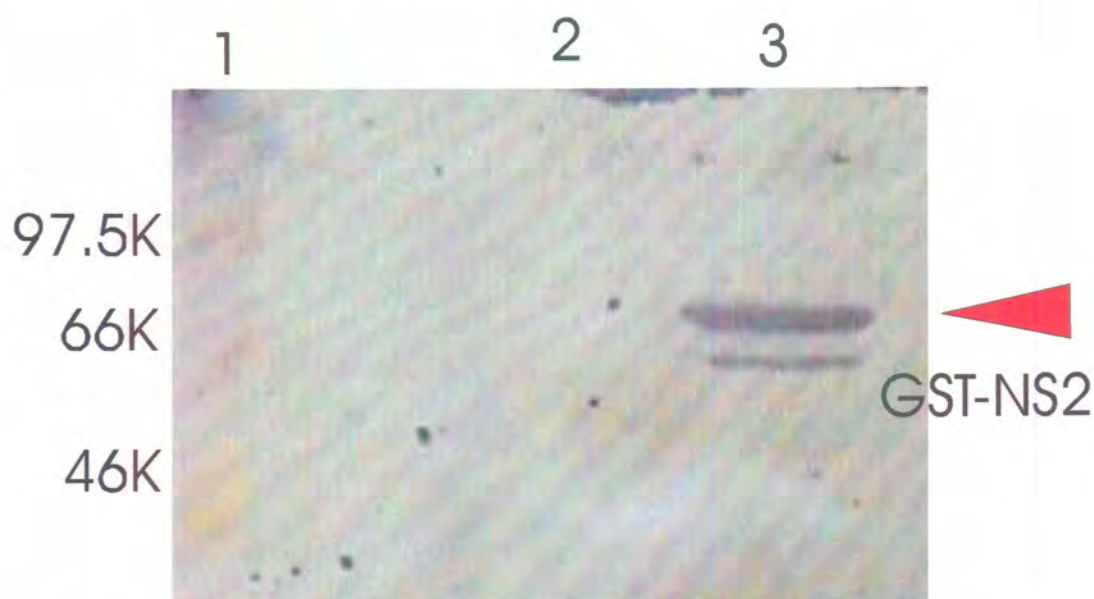


Fig. 3.6: Western blot analysis indicating prokaryotic expression of the BTV GST-NS2 fusion protein. Molecular weights of the protein marker (AEC Amersham), in kilodalton, are indicated (lane 1). Lane 2, *E. coli* cells transformed with the pGEX vector expressing GST protein; lane 3, *E. coli* cells transformed with the pGEX-NS2 vector expressing a GST-NS2 protein of 69 kDa, as indicated by the arrows.

3.3.3 RNA-protein binding experiments using the IAsys biosensor

Crude *E. coli* cell lysates were used as a source of NS2 protein for the RNA-protein binding experiments. Although native *E. coli* proteins were also present in the extract together with the NS2 protein, it would not affect the RNA-protein binding analysis as the experimental procedure used, would select only for GST-tagged proteins by first binding them to a GSH protein. Therefore, only GST-NS2 proteins will stay bound during the RNA-protein binding experiment and all other proteins would be washed away. The NS2-GST protein was immobilized onto the surface of a CMD cuvette in an indirect manner by first immobilizing an AHA spacer followed by binding of GSH to the spacer molecules and then binding of the NS2-GST protein to the previously bound GSH protein. Data

collected during the indirect immobilization of the NS2-GST protein to the matrix are presented in Fig. 3.7 and Table 3.1.

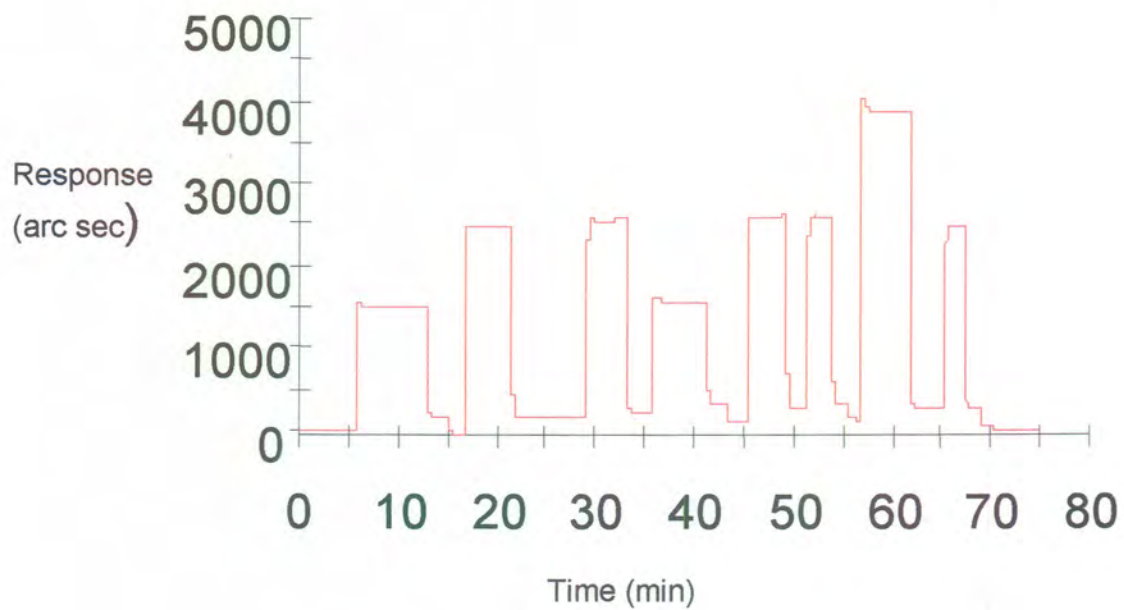


Fig. 3.7: Graphical presentation of the different stages involved in preparing the CMD cuvette for use in the IAsys biosensor to study RNA-protein binding. The individual steps are clarified in Table 3.1.

Table 3.1: Details of the events using the IAsys biosensor to study RNA–protein binding

Event type**	Experiment time (min)	Response (Arc seconds)*
Baseline with PBS-Tween	0 - 5.76	7
Activation with EDC and NHS	5.76 – 12.85	1489.75
PBS-Tween wash	12.85 – 15.20	177.37
Change to 0.15 M STE	15.20 – 16.88	0
Add AHA spacer	16.88 – 21.55	2444
PBS- Tween wash	21.55 – 28.87	182.70
Blocking step with 1 M ethanolamine	28.87 – 33.31	2493.81
PBS-Tween wash	33.31 – 35.69	211.65
Activation with EDC and NHS	35.69 – 41.40	1560.97
PBS-Tween wash	41.40 – 43.27	326.26
Change to 0.15 M STE	43.27 – 45.35	96.80
Add GSH protein	45.35 – 49.33	2588.37
PBS-Tween wash	49.33 – 51.41	314.32
Blocking step with 1 M ethanolamine	51.41 – 54.02	2540.88
PBS-Tween wash	54.02 – 55.68	354.99
Change to 0.15 M STE	55.68 – 56.96	135.12
Add BTV NS2 fusion protein	56.96 – 61.74	3872.04
PBS-Tween wash	61.74 – 65.25	292.99
Blocking step with 1 M ethanolamine	65.25 – 67.41	2405.31
Change to 0.15 M STE	67.41 – 69.07	270.94
Add RNA transcript	69.07 – 76.41	24.55

* The arc seconds value is the average over the time period indicated.

** The same events are indicated in the same colour (events are explained on the next page)

The results obtained indicated that the NS2-GST fusion protein was successfully immobilized on the CMD cuvette. The results, as indicated in Table 3.1, can be interpreted in the following manner.

Baseline data with PBS-Tween:

This step is to equilibrate the cuvette before starting to collect data. Baseline data was collected for 5 min with an arc sec value of almost zero.

PBS-Tween wash:

After every step in the experimental procedure, a salt wash was done to remove reagents and unbound molecules.

Activation with EDC and NHS:

The CMD surface size is 200 nm and consists of dextran, a polymer of glucose residues. It is carboxymethylated to give approximately one carboxyl group per two glucose residues ($pK_a = 3.5$). The CMD surface will be negatively charged in buffers with a $pH > 5$. If the buffer pH is less than the isoelectric point (pI) of a protein, the protein will have a net positive charge and will bind to the carboxylate groups of the matrix via amino groups of the protein. An increase in the refractive index (1489.75 arc sec) that will stabilize at a higher level was observed. After the wash steps were completed, the reading returned to slightly above the previous value (177.37 arc sec).

Change to 0.15 M STE:

The proteins GSH and BTV GST-NS2 as well as the AHA spacer molecules were prepared in a 0.15 M STE buffer. Before immobilizing these proteins to the cuvette, the cuvette was thus equilibrated with the buffer.

Add AHA spacer

After adding the AHA spacer there was an increase in the refractive index that stabilized at 182.70 arc sec after washing with PBS-Tween. This indicated that 0.92 ng protein/ mm^2 sensor surface was bound.

Blocking step with 1 M ethanolamine:

During the blocking step all unreacted charged groups on the CMD matrix were blocked. This led to an increase in the refractive index and after washing with PBS-Tween the reading returned to the previous value.

Add GSH protein:

After adding the GSH protein there was an increase in the refractive index that stabilized at 314.32 arc sec after washing with PBS-Tween. This indicated that 1.7 ng protein /mm² sensor surface was bound.

Add BTV NS2 fusion protein:

After adding the BTV NS2 fusion protein there was an increase in the refractive index that stabilized at 292.99 arc sec after washing with PBS-Tween. This indicated that 1.22 ng protein /mm² sensor surface was bound.

Add RNA transcript:

After adding the RNA transcript, the arc sec value was 24, indicating that RNA did not bind sufficiently to the NS2 protein or that binding could not be measured because it occurred outside the evanescent field.

For screening applications, 200 arc seconds corresponding to 1 ng protein/mm² sensor surface are sufficient for analysis. Immobilization levels should be as low as possible to minimize steric hindrance (<http://www.affinity-sensors.com/>). In this study 0.92 ng protein/mm² AHA spacer, 1.7 ng protein/mm² GSH protein and 1.22 ng protein/mm² BTV NS2 fusion protein were immobilized to the CMD matrix, indicating sufficient levels of bound protein. The difference in the amount of bound protein may be because unequal amounts of protein were added to the matrix.

3.4 DISCUSSION

A new method whereby RNA-protein binding can be quantified and which does not require the use of radioactively labelled RNA or purification of the protein was investigated. Different transcription vectors were constructed which contained the full-length segment 8 gene of BTV, EHDV and AHSV under control of the T7 RNA polymerase promoter. *In vitro* RNA transcripts were generated to investigate whether or not BTV NS2 binds preferentially to BTV-specific mRNA. The use of the IAsys biosystem to quantify differences in the binding ability of BTV NS2 to the respective orbivirus mRNAs was investigated.

The IAsys system allows molecular interactions to be monitored quantitatively (<http://www.affinity-sensors.com/>). This is achieved by using an evanescent field to

measure minute changes in the refractive index and thickness at the surface of a resonant mirror. Binding can be seen as shifts in the resonance angle if the RNA in free solution binds to a protein immobilized on the surface of the sensor. Cuvettes are available with a variety of sensor surfaces. For this study, a carboxymethyl dextran (CMD) surface was chosen which consists of a hydrophilic three-dimensional matrix containing charged carboxylate groups. The CMD surface is negatively charged in buffers used with a pH > 5. If the pH of the buffer is less than the isoelectric point of a protein, the protein will exhibit a net positive charge and it will bind to the matrix by electrostatic interaction. The interaction involves binding of the protein's amino groups to the carboxylate groups of the CMD matrix by using EDC and NHS chemistry.

Rubio *et al.* (1997) reported an assay whereby the interactions between a GST protein and a second protein could be investigated by using the IAsys biosystem. Their results indicated that immobilization of the ligand should occur in such a manner that the binding activity of the protein is retained. Direct immobilization of the GST fusion protein to the CMD matrix resulted in a loss of binding activity. This could be due to conformational changes in the protein that influence its binding activity or the binding sites being blocked. To overcome this problem, they have proposed a strategy for indirect immobilization of the ligand where a spacer is first bound to the CMD matrix, then a GSH protein and finally the GST-fusion protein. Their approach was modified in this study by adding RNA to the immobilized GST-NS2 in order to investigate RNA-protein binding interactions. RNA binding to the GST-fusion protein coupled to GSH could not be established. However, if binding has indeed occurred, detection may have been impossible if this binding interaction occurred outside of the evanescent field. The evanescent field extends 200 nm from the sensor surface and decays exponentially away from the surface. Therefore, only interactions close to the sensor surface can be monitored.

Despite the lack of detectable RNA-protein binding, the IAsys system could nevertheless be useful in the study of RNA-protein interactions. It potentially represents a rapid method that can yield both qualitative and quantitative data without the need for radioactively labelling the RNA. There is also no need for the NS2-GST fusion proteins to be purified. Problems identified during the course of this study may be overcome by further investigating ways of binding the protein to a matrix directly without losing its

binding ability. This will ensure that when RNA is added to the protein the binding will still occur in the evanescent field where it can subsequently be detected. The development of a matrix for the direct immobilization of GST-fusion proteins, without having to use a spacer and GSH protein, should also facilitate such RNA-protein binding studies. Thus, although this technique may appear to be promising, further research should be performed to refine the methodology for application to RNA-protein binding analysis.

CHAPTER 4:
SECONDARY STRUCTURE ANALYSIS OF THE mRNA SEGMENTS OF DIFFERENT
ORBIVIRUSES AND IDENTIFICATION OF STRUCTURAL DETERMINANTS THAT
MAY BE INVOLVED IN RNA-PROTEIN BINDING

4.1 INTRODUCTION

Many biological processes rely on specific interactions between RNA and protein molecules. In segmented dsRNA viruses the individual RNA segments are sorted and packaged to ensure that only one copy of each segment is packaged per virus particle. RNA molecules have a distinctive secondary structure and these recognition events may be mediated by the conformation of the RNA. Structural motifs consisting of conserved terminal sequences and segment-specific inverted repeats is common to the segmented genomes of members of the *Reoviridae* (Joklik, 1981; Antczak *et al.*, 1982; Anzola *et al.*, 1987). In wound tumor virus (WTV), part of the *Phytoreovirus* genus in the family *Reoviridae*, it was proposed that a base-paired structure involving the terminal inverted repeats and the adjacent stem-loop structures within the 3' terminal domain, could serve as a structural signature to distinguish one segment from another (Xu *et al.*, 1989). For BTV, the prototype virus in the orbivirus genus in the family *Reoviridae*, no specific nucleotide sequence on the viral RNA that may be involved in packaging of the ten different RNA segments was previously identified. It may be that a specific secondary structure on the RNA may be responsible for this packaging process. In this study the secondary structures of the ten different mRNA segments of BTV-10, mutant mRNA transcripts where the 5' or/and 3' inverted repeat sequences were removed and different orbivirus segment 8 RNAs were calculated and unique and conserved secondary structures that may serve as sorting signals were identified.

The secondary structures of BTV-10 RNA segments have previously been analysed with the use of the "FOLD" program (Cowley *et al.*, 1992). This program could accommodate only full-length transcripts of genomic segments 7-10. Transcripts from the larger genomic segments 1-6 were modified to contain only 1200 nucleotides by splicing together 600 nucleotides each from the 5' and 3' terminal ends, respectively. Cowley *et al.* (1992) also reported that base-pairing did occur between the 5' and 3' inverted repeat sequences and in some of the RNA segments the conserved hexanucleotide sequences were included in this structural motif. One or two conserved stem-loop structures near the 3' end of segment 2, 3,4,5,7,8, and 10 were identified. In this study, full-length sequences of all ten BTV-10 segments were used to predict secondary structures, thereby resulting in a more accurate prediction of the structure and identification of conserved stem-loop structures. The hexanucleotide sequences were also not involved in the base-pairing structure of the inverted repeat sequences.

The secondary structures of different RNAs were predicted in this study using the RNADraw program as described by Matzura *et al.* (1996). In this program the primary structure of RNA is the list of covalently linked nucleotides from the 5' to 3' end and the secondary structure is the list of base pairs, denoted by i - j for a pairing between the i^{th} and j^{th} nucleotides, r_i and r_j , where $i < j$. Also, A-U and G-C base-pairing are assumed and weaker G-U base-pairing (canonical base-pairing) is assumed where the bases bind in a skewed fashion. The number of theoretically possible foldings of a given strand can be quite large, although some foldings are preferred over others. This preference is determined by the thermodynamic properties of the molecule. Stacked regions stabilize the structure while single-stranded sections (loops) destabilize it. Turner *et al.* (1988) formulated rules that enable the determination of stabilizing and destabilizing characteristics of each of these substructures. The tendency is for a molecule to seek out the most stable structure, a tendency that may be expressed in minimization of its global free energy. RNA-folding algorithms attempt to find the structure that represents the minimum global free energy of a molecule. Free energies are assigned to base pair stacks and to loops and are summed to calculate the overall free energy difference of folding. Base pair stacks take into account both hydrogen bonds and stacking effects. Loop energies comprise an entropic term for loss of conformational freedom and other terms that take into account mismatched pair stacking, co-axial helix stacking, single base pair stacking and empirically derived corrections.

The genome of RNA viruses evolves very quickly due to a high mutation rate per nucleotide. As far as the importance of RNA secondary structure is concerned, such higher order structure may contain conserved features even if the nucleotide sequence may be variable (Hofacker *et al.*, 1998). By comparing the RNA secondary structures of different orbiviruses viruses, conserved structures may be identified. These conserved structures may play a role in the selection and packaging of orbiviral mRNA segments into precursor viral particles. The nucleotide sequences of segment 8 of different orbiviruses have already been determined (Table 4.1) and these sequences were used to identify conserved secondary structures using the programs ALIDOT and PFRALI (Hofacker *et al.*, 1998). The procedure and algorithms are further discussed in the methods section.

After performing RNA-NS2 binding studies using mutant transcripts lacking inverted repeat sequences, the results indicated that these sequences might influence binding indirectly through an alteration in the secondary structure of the RNA (Chapter 2). RNA secondary structure may therefore play a role in the packaging of the ten different BTV mRNA segments into a viral particle. To investigate, computer-based secondary structure predictions of these mutant transcripts were compared to the full-length segment 8 mRNA. In addition, secondary structure analysis of the segment 8 RNA of different orbiviruses was carried out to identify conserved secondary structures that may be involved in non-specific binding. Since gene reassortment has not yet been observed in cells simultaneously infected with BTV and EHDV, it is possible that features that are unique to the RNA of a particular orbivirus, may exist on different orbivirus RNAs. Unique secondary structures that may be involved in specific binding of the RNA to the NS2 protein were manually identified following comparative analysis of the respective secondary structures.

4.2 MATERIALS AND METHODS

4.2.1 GenBank accession numbers

The sequences used in the secondary structure analysis may be accessed with the following GenBank codes:

Table 4.1: GenBank accession numbers of sequences used in the secondary structure analysis

Sequence	GenBank accession number
BTV2 – VP1 (Segment 1)	X12819
BTV10 - VP2 (Segment 2)	M11787
BTV10 - VP3 (Segment 3)	M22096
BTV10 - VP4 (Segment 4)	Y00421
BTV10 - VP5 (Segment 5)	D12532
BTV10 - VP6 (Segment 9)	L08669
BTV10 - VP7 (Segment 7)	X06463
BTV10 - NS1 (Segment 6)	Y00422
BTV10 - NS2 (Segment 8)	X13374
BTV10 - NS3 (Segment 10)	AF044372
AHSV9 - NS2	M69090
EHDV2 - NS2	M69091
KASV – NS2	AB018090

4.2.2 Computational analysis of RNA secondary structures

The secondary structures of mutant transcripts lacking inverted repeat sequences, i5'3'BTVNS2, i5'BTVNS2 and i3'BTVNS2 (Chapter 2), the ten BTV-10 RNA segments as well as the segment 8 RNA of AHSV, EHDV and Kasba virus (KASV) were calculated. The sequences were obtained from GenBank (accession numbers in Table 4.1) and calculations were performed using RNADraw V1.1 for Windows 95 (Matzura and Wennborg, 1996). The optimal base pair probability matrix curve calculation algorithms were all ported directly from the Vienna RNA package V1.1 (Hofacker *et al.*, 1994). The implemented dynamic programming algorithm is based on work by Zuker and Stiegler (1981). The base pair probability algorithm is based on work by McCaskill (1990). Energy parameters were taken from the work of Turner *et al.* (1988), Freier *et al.* (1986), Jaeger *et al.* (1989) and He (1991). The secondary structure coordinates were calculated with NAVIEW (Bruculeri and Heinrich, 1988), ported by permission. This program is particularly suitable for displaying large sequences and automatically generates a structure with very limited overlap of strands. Minimum free energy

calculations were performed without forcing the 5' and 3' ends of the RNA transcripts to base pair. All other program parameters were kept at the default values.

4.2.3 Detection of conserved RNA structural elements in the segment 8 RNAs of different orbiviruses

The programs ALIDOT and PFRALI (Hofacker *et al.*, 1998), part of the Vienna RNA Package (Hofacker *et al.*, 1994), were used to detect conserved secondary structures in small data sets of related RNA sequences. An alignment of different orbivirus segment 8 sequences was obtained without any reference to the predicted secondary structure using CLUSTALW (Thompson *et al.*, 1994). Base-pairing probabilities, as obtained from McCaskill's partition function algorithm (McCaskill, 1990), were displayed as dot plots. A combined dot plot of the probability matrixes of the individual sequences was generated. The program ALIDOT detected conserved structures using the multiple sequence alignments and minimum energy structure predictions. A secondary structure, indicating all the conserved structural characteristics, were predicted by the PFRALI program. A flow diagram of how this program operates, is indicated in Fig. 4.1.

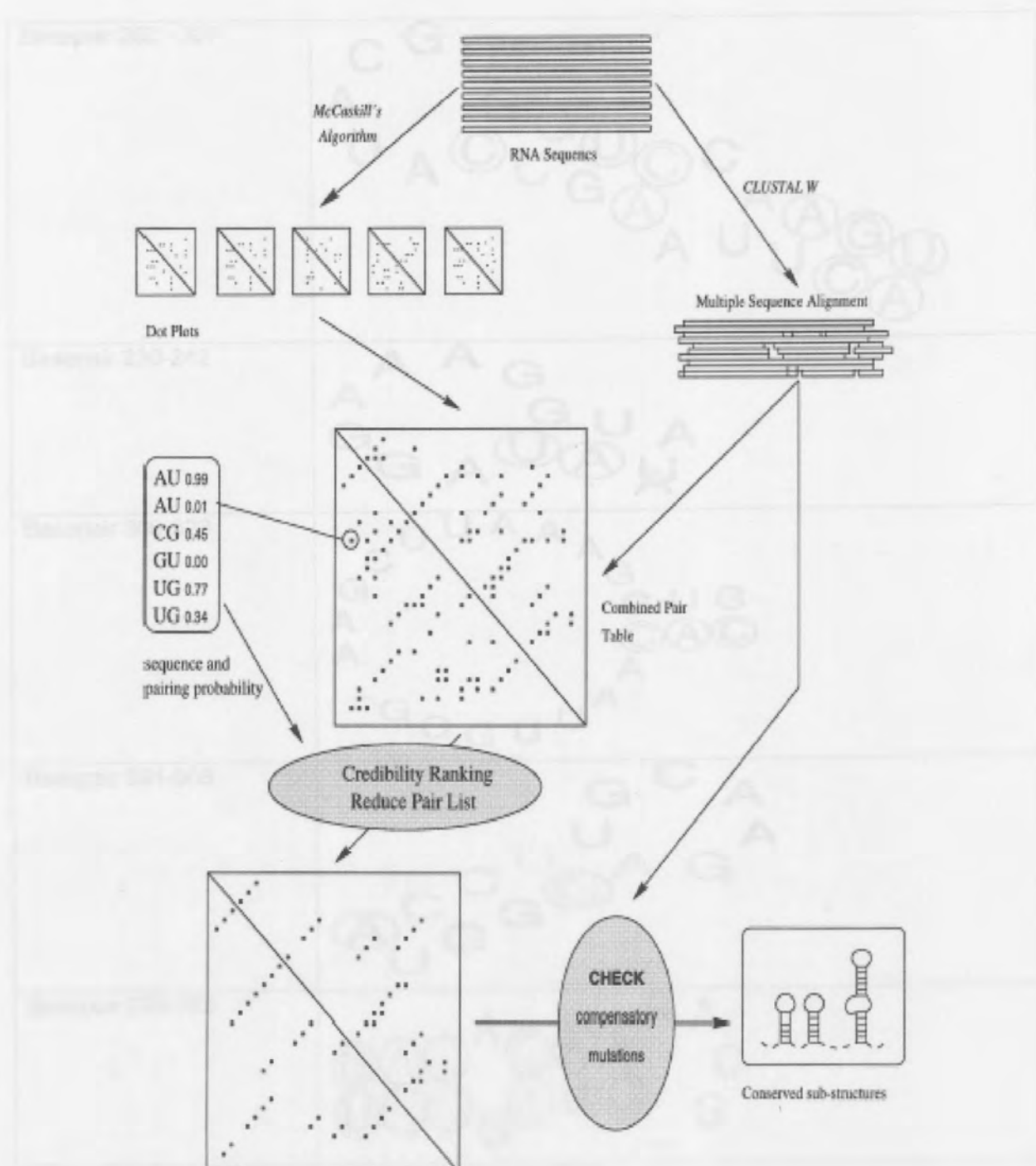


Fig. 4.1: Diagrammatic representation of the comparison of different secondary structures using the programs ALIDOT and PFRALI (Hofacker *et al.*, 1999).

4.3 RESULTS

4.3.1 Secondary structure analysis of the full-length mRNA segments of BTV-10

The secondary structure analysis of the ten full-length mRNA transcripts of BTV-10 indicated that base-pairing at the inverted repeat sequences does occur in each of the RNA transcripts (Fig. 4.2 – 4.3). The secondary structure predictions of the full-length BTV mRNA transcripts are indicated in Fig. 4.4 to Fig. 4.8. From the analysis it is evident that the repeats play a crucial role in the mature BTV RNA transcripts. A feature common to each transcript was the presence of one or two stem-loop structures at either the 5' or 3' end of all ten BTV mRNA transcripts adjacent to the inverted repeat sequences (Fig. 4.2 – 4.3). These structures consist of a base-paired stem structure that differs in length between the different RNA segments and ends in a loop. On BTV RNA segments 1-8 only one stem-loop structure adjacent to the inverted repeat base-paired sequences were identified, but on segment 9 three different stem-loops were identified and on segment 10 two stem-loop structures were present. The functional significance of these structures awaits further mutational analysis.

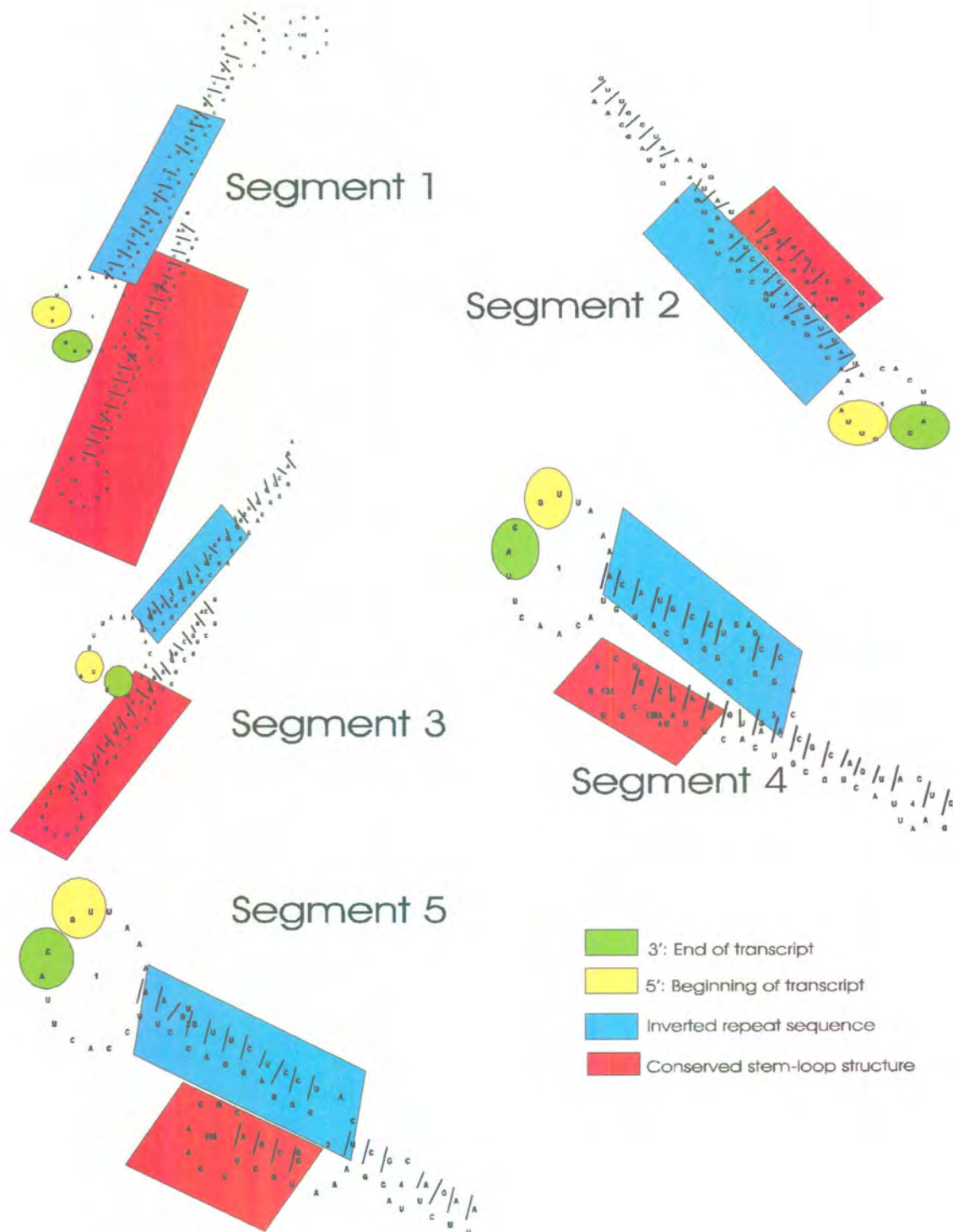


Fig. 4.2: Magnification of the 5' and 3' ends of BTV-10 mRNA segments 1-5, indicating the base-pairing at the inverted repeat sequences and the conserved stem-loop structure present at the 5' or 3' ends of all ten mRNA transcripts

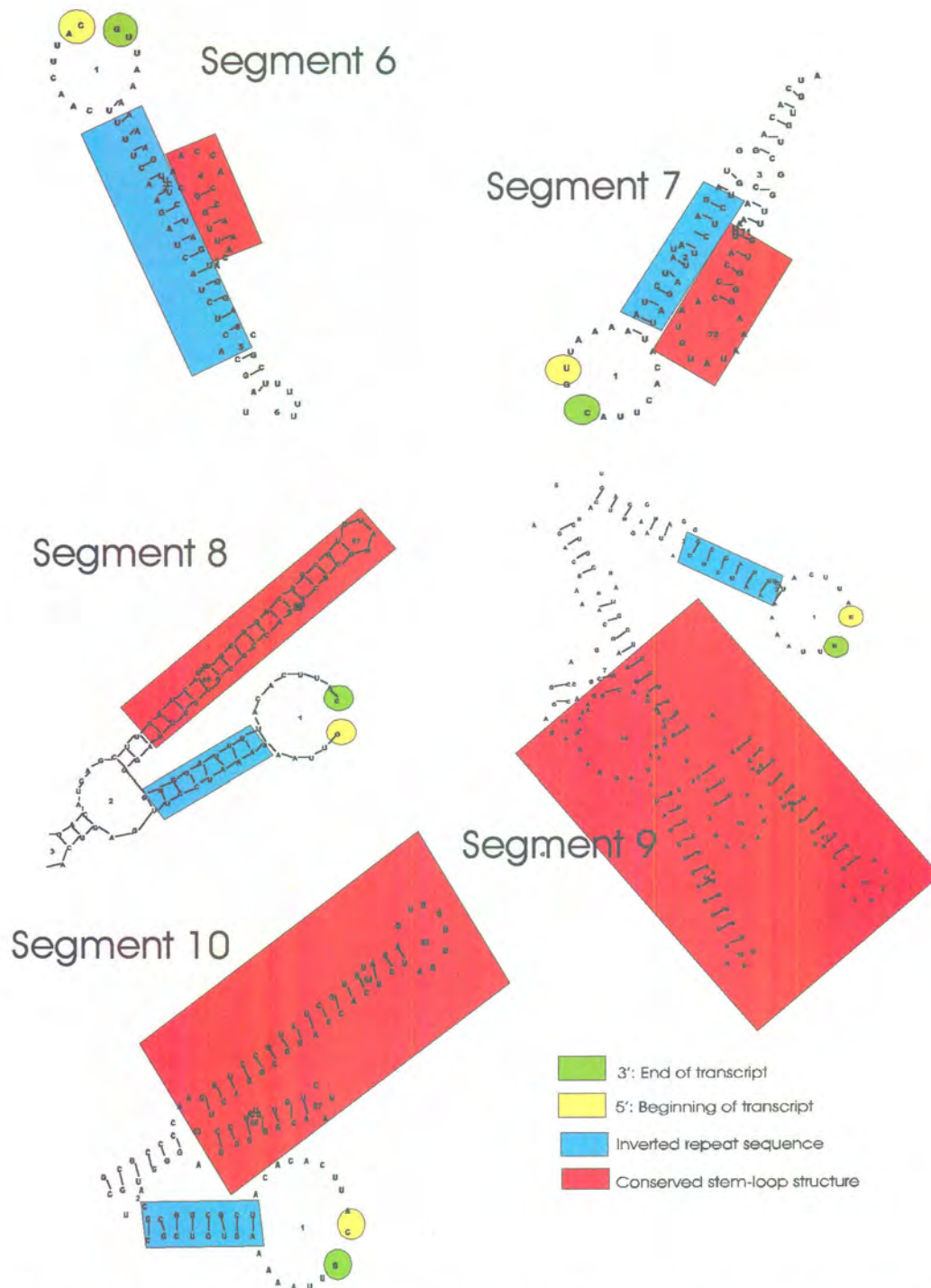


Fig. 4.3: Magnification of the 5' and 3' ends of BTV-10 mRNA segments 6-10, indicating the base-pairing at the inverted repeat sequences and the conserved stem-loop structure present at the 5' or 3' ends of all ten mRNA transcripts.

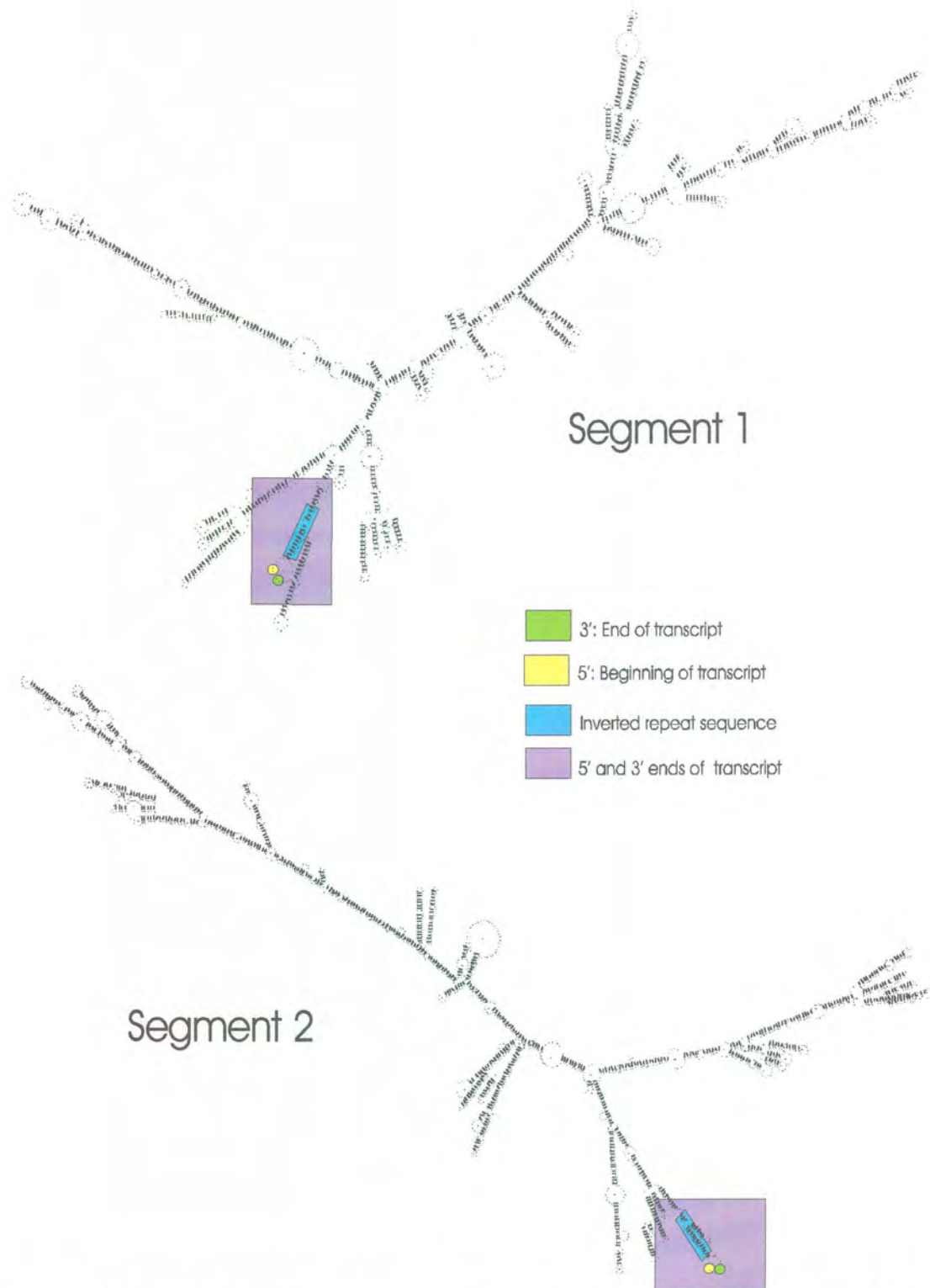


Fig. 4.4: Secondary structure prediction of full-length RNA segments 1 and 2 of BTV-10 indicating base-pairing at the 5' and 3' inverted repeat sequences.

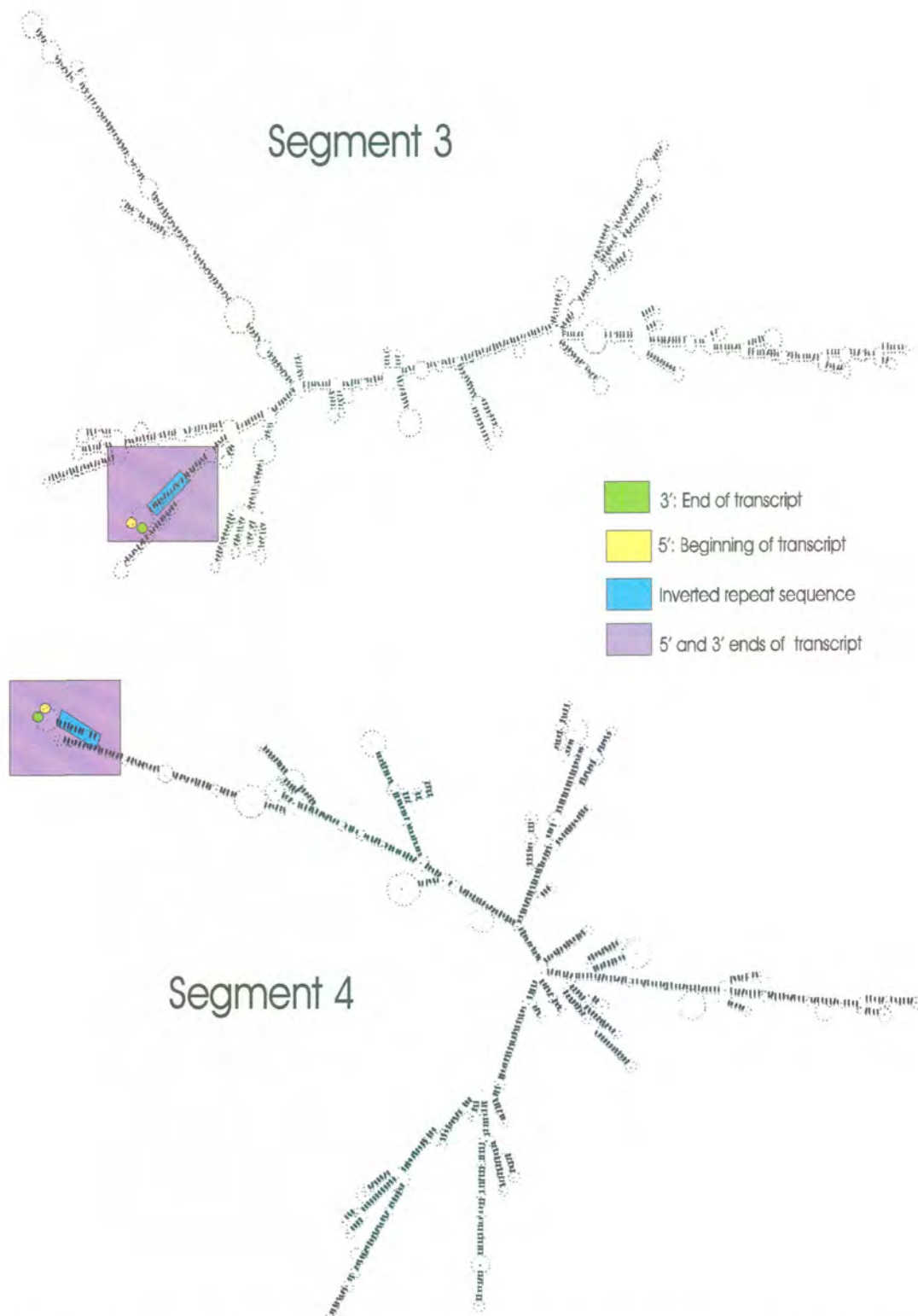


Fig. 4.5: Secondary structure prediction of full-length RNA segments 3 and 4 of BTV-10 indicating base-pairing at the 5' and 3' inverted repeat sequences.

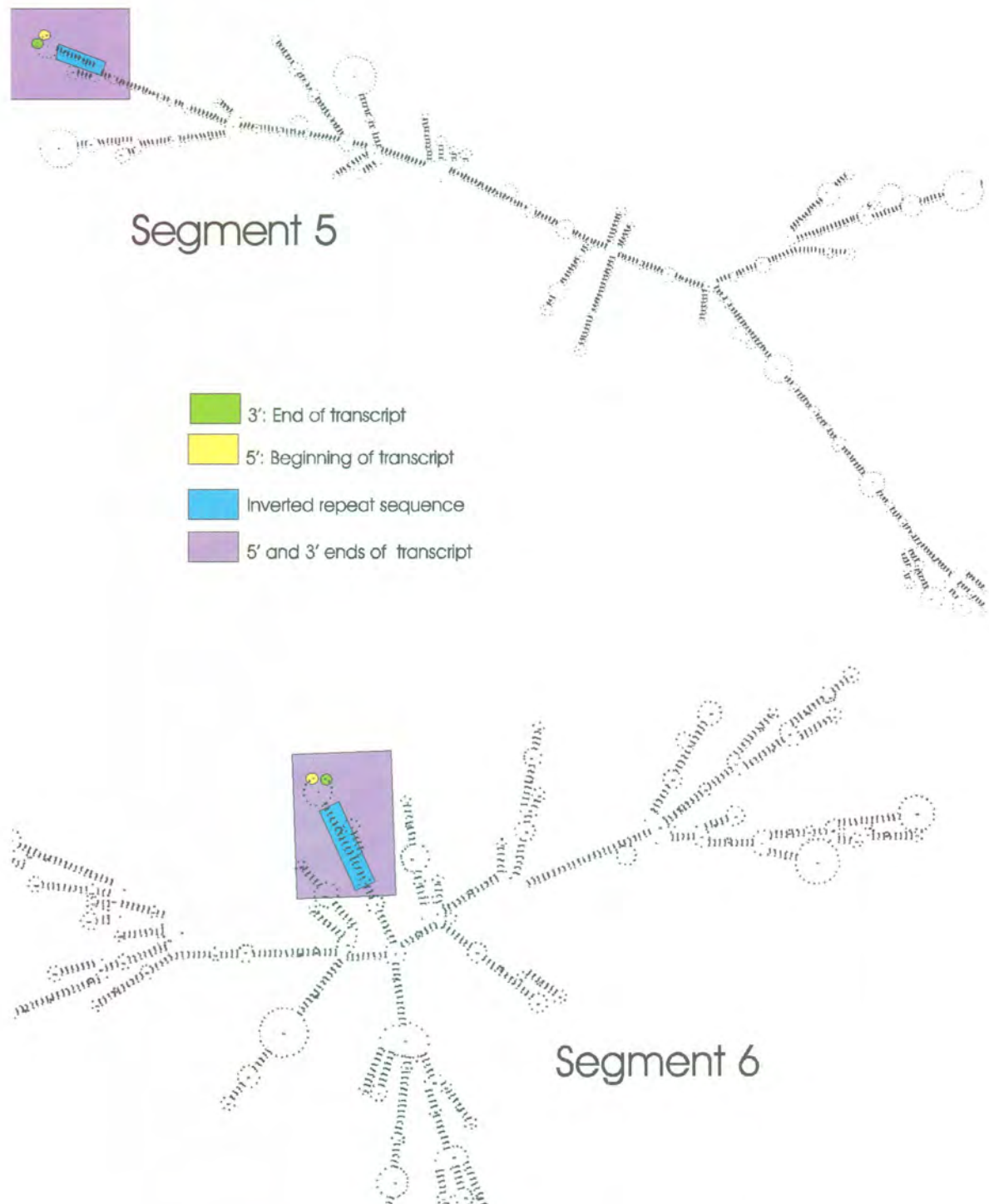


Fig. 4.6: Secondary structure prediction of full-length RNA segments 5 and 6 of BTV-10 indicating base-pairing at the 5' and 3' inverted repeat sequences.

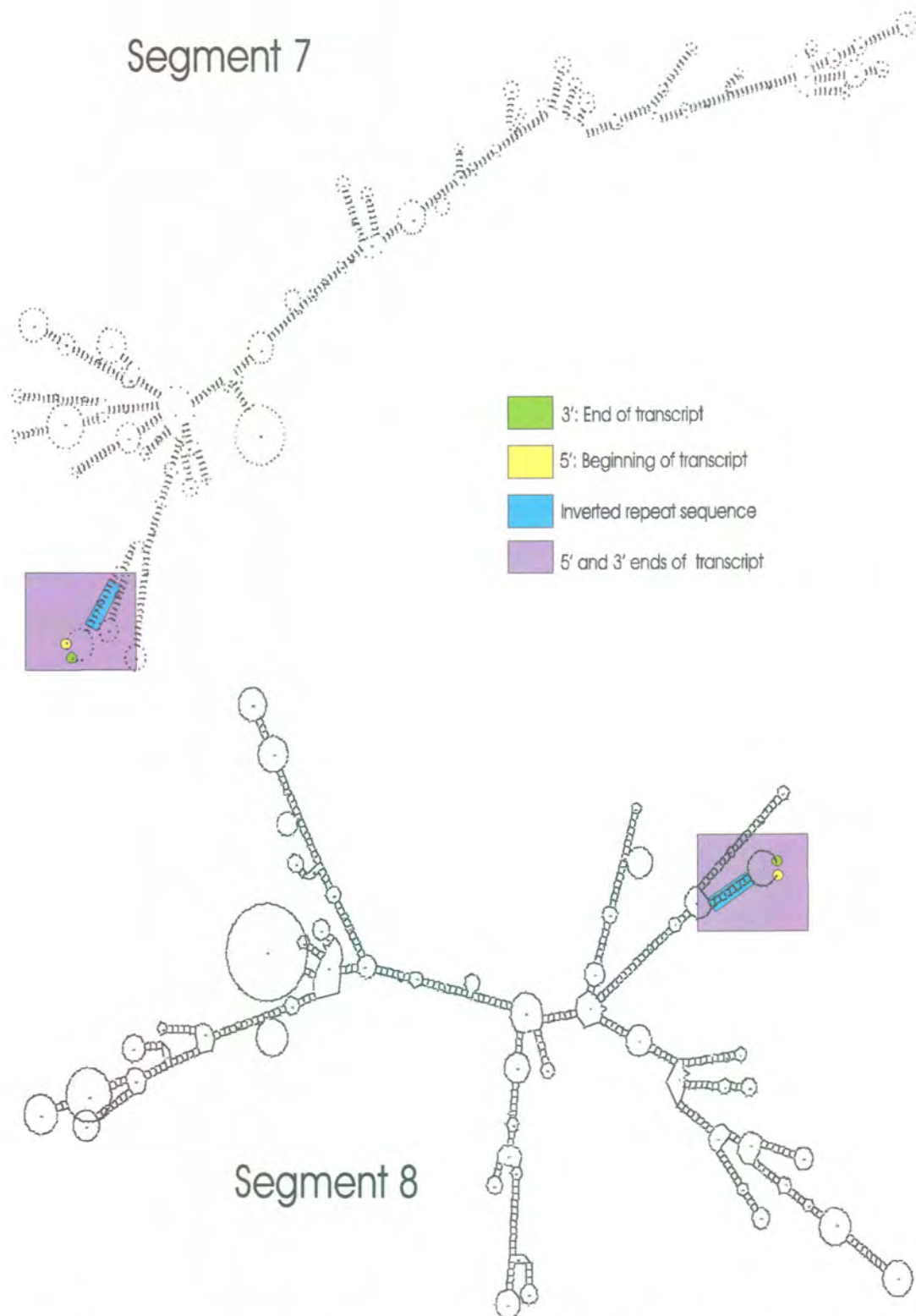


Fig. 4.7: Secondary structure prediction of full-length RNA segments 7 and 8 of BTV-10 indicating base-pairing at the 5' and 3' inverted repeat sequences.

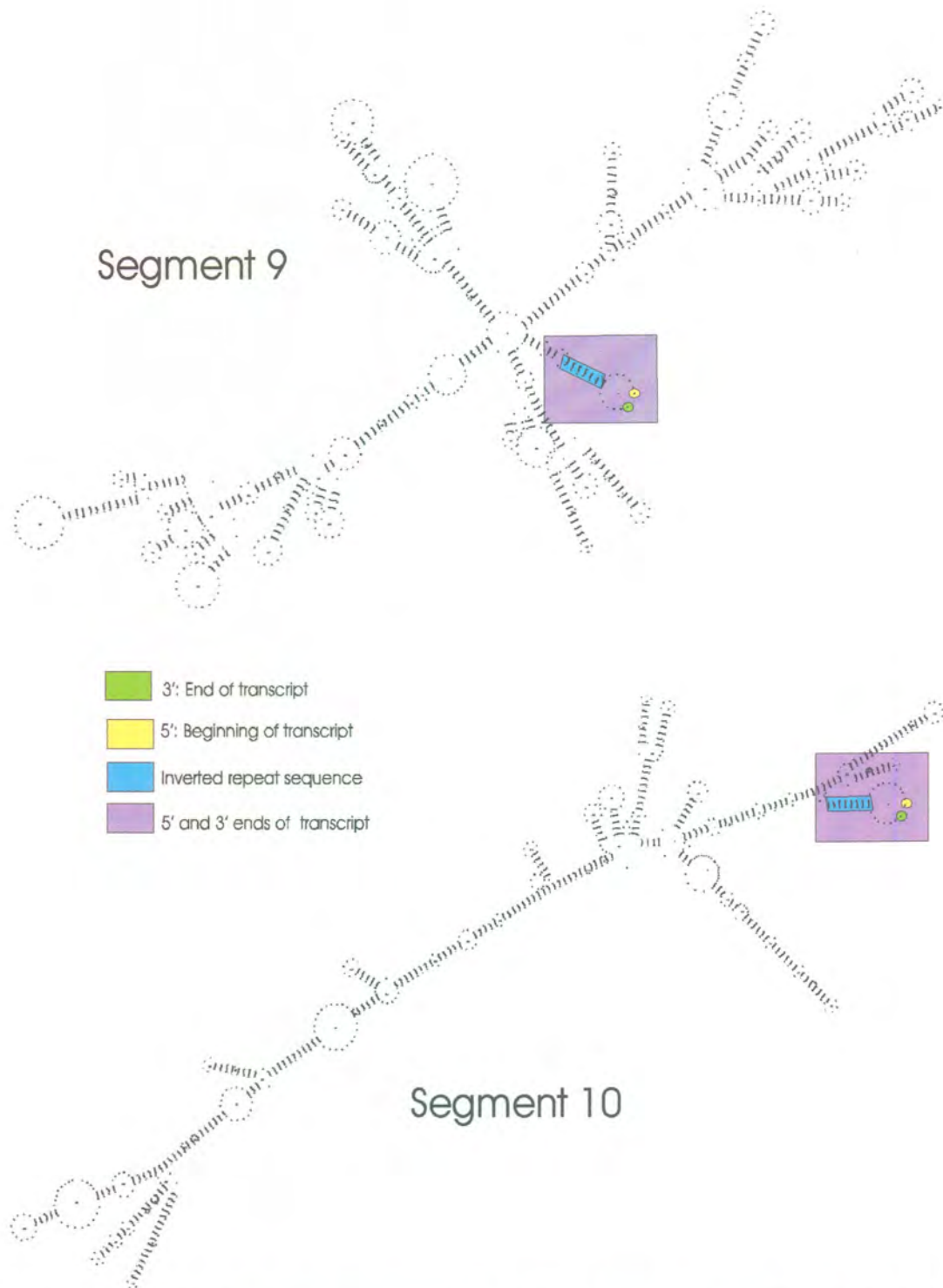


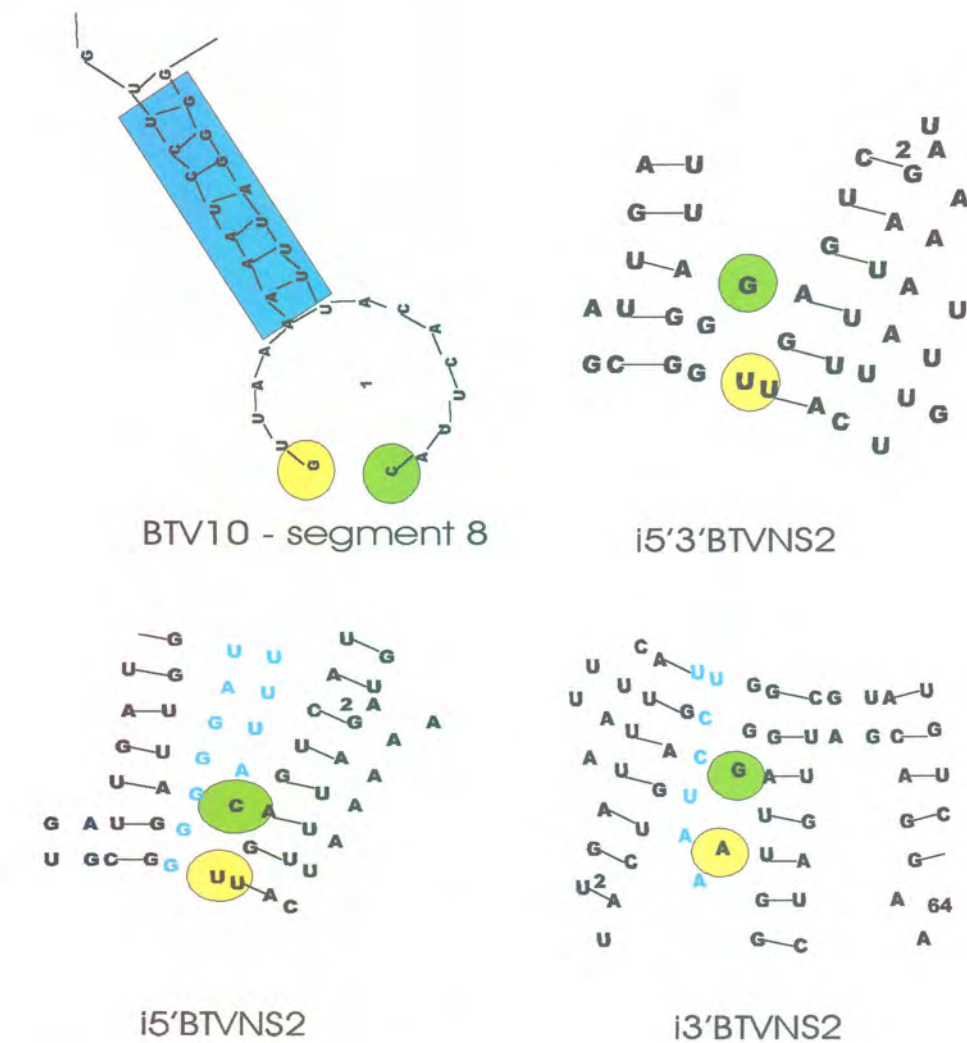
Fig. 4.8: Secondary structure prediction of full-length RNA segments 9 and 10 of BTV-10 indicating base-pairing at the 5' and 3' inverted repeat sequences.

4.3.2 Secondary structure predictions of the mutant transcripts lacking inverted repeat sequences

Secondary structures of the full-length BTV-10 segment 8 RNA and deletion mutants lacking the 5' and/or 3' inverted repeat sequences (Chapter 2) were determined. Results indicated that the deletion of the inverted repeat sequences led to a widespread disruption of the secondary structure (full-length sequences indicated in Fig. 4.10 – Fig. 4.13) due to the absence of 5' or 3' complementarity. In the full-length BTV-10 segment 8 RNA, 334 base pairs participated in the secondary structure. However, in the mutant RNA transcripts analysed, only 309 base pairs were observed. It is therefore possible that the inverted repeat sequences may play an important role in maintaining the overall secondary structure of the full-length BTV RNA transcript.

In Chapter 2, RNA-protein binding studies indicated that removal of the inverted repeat sequences led to a decline in RNA-NS2 binding. It was proposed that alterations in the secondary structure of the RNA might be responsible for the observed reduction in binding of the mutant transcripts to NS2. In predicting the secondary structures for these mutant transcripts, it was evident that removal of the inverted repeat sequences led to a cascade effect in which the mutant transcripts displayed higher order structures which were very different to that of the full-length parental BTV segment 8 RNA transcript. Mutant RNA transcripts in which both the 5' and 3' inverted repeat sequences were removed, demonstrated a lesser decline in binding than the mutant transcripts in which only the 5' or 3' inverted repeat sequence was removed. No differences in the overall secondary structure of these RNA transcripts was observed, except for changes in the 5' and 3' terminal regions (Fig. 4.9). The mutant transcripts i5'BTVNS2 and i3'BTVNS2 displayed similar binding affinities to the NS2 protein. Secondary structure analysis indicated that both the transcripts contained a single-stranded region at the 3' or 5' ends which is absent in the secondary structure of i5'3'BTVNS2. The NS2 protein displayed a higher binding affinity for the latter transcript compared to those in which only the 5' or 3' inverted repeat sequences were deleted. The single-stranded regions may therefore influence the binding affinity of the transcripts to the BTV NS2 protein by masking the potential binding sites. Although the mutant RNA transcripts used in the RNA-protein binding studies in Chapter 2 contained additional vector sequences and the 5' and 3' hexanucleotide sequences were also removed, secondary structure analysis of these RNA transcripts indicated that they did not differ from the structures presented in Fig.

4.10 to Fig. 4.13 in which only the inverted repeat sequences have been deleted (data not shown).



- 3': End of transcript
- 5': Beginning of transcript
- Inverted repeat sequence

Fig. 4.9: Magnification of the 5' and 3' terminal secondary structures of mutant transcripts lacking inverted repeat sequences.

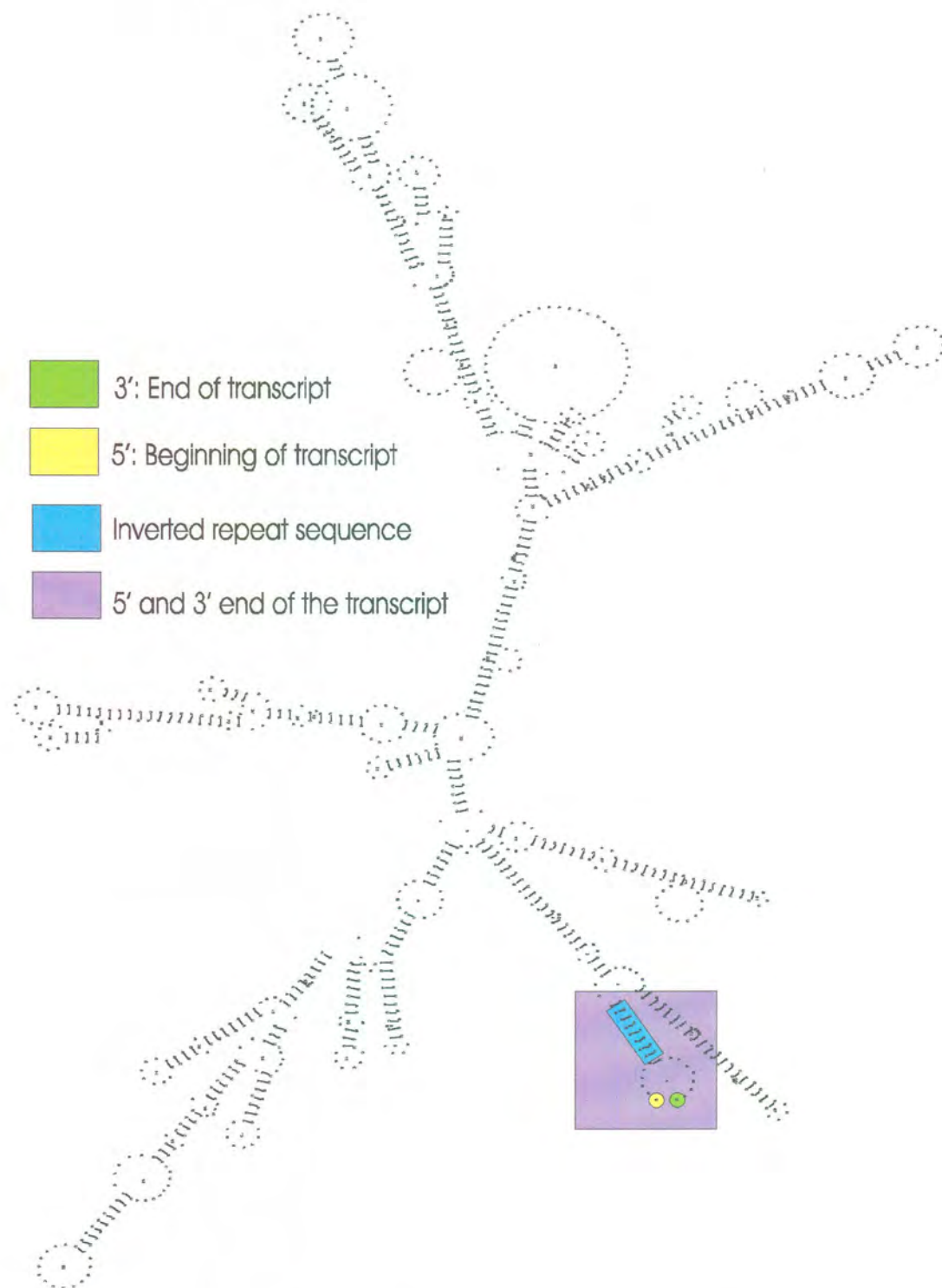


Fig. 4.10: Secondary structure analyses of the full-length BTV 10 segment 8 RNA transcript.

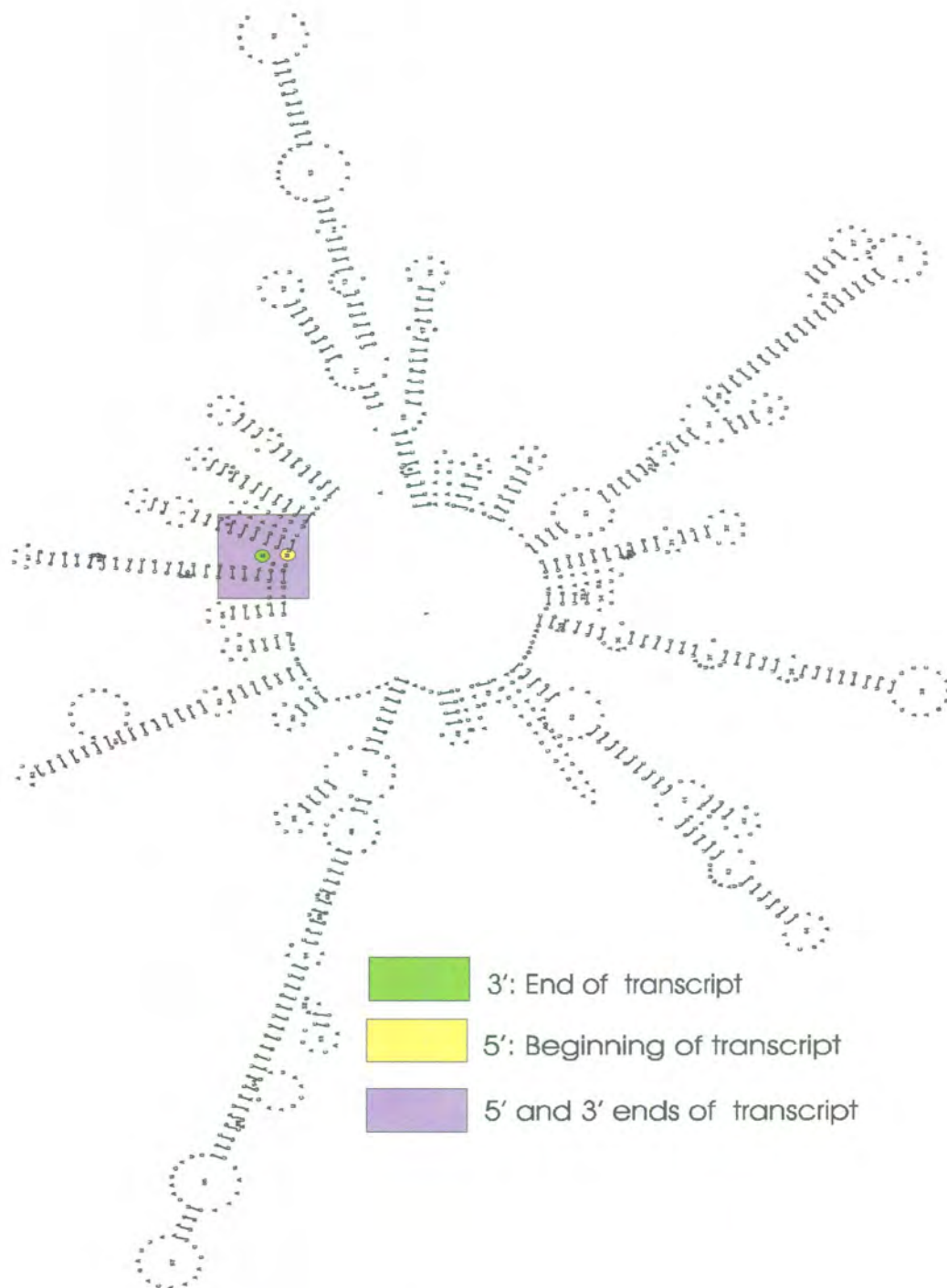


Fig. 4.11: RNA secondary structure prediction of the mutant transcript i5'3'BTVNS2 from which both the 5' and 3' inverted repeat sequences were removed.

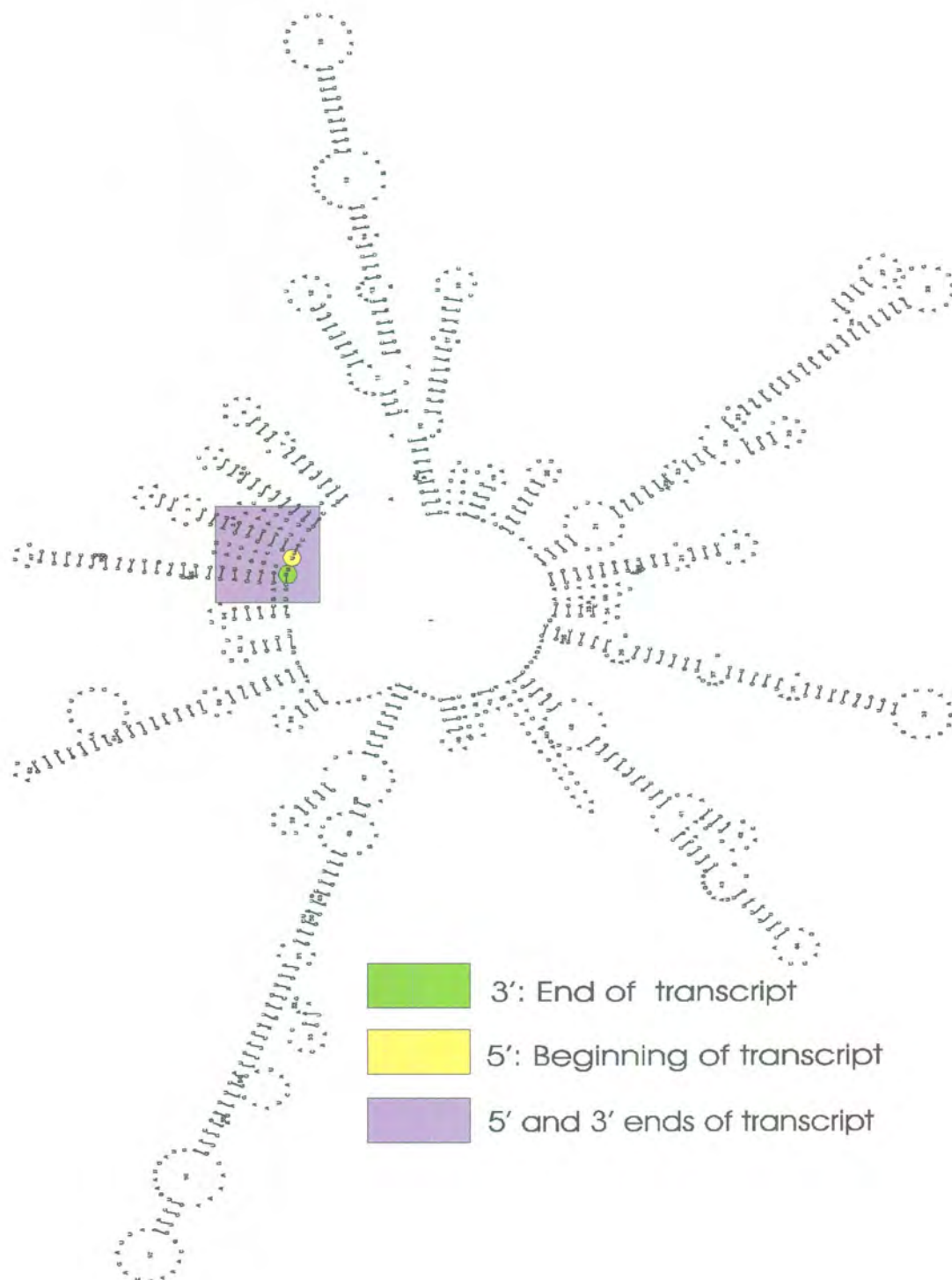


Fig. 4.12: RNA secondary structure prediction of the mutant transcript i5'BTVNS2 from which the 5' end inverted repeat sequence was removed.

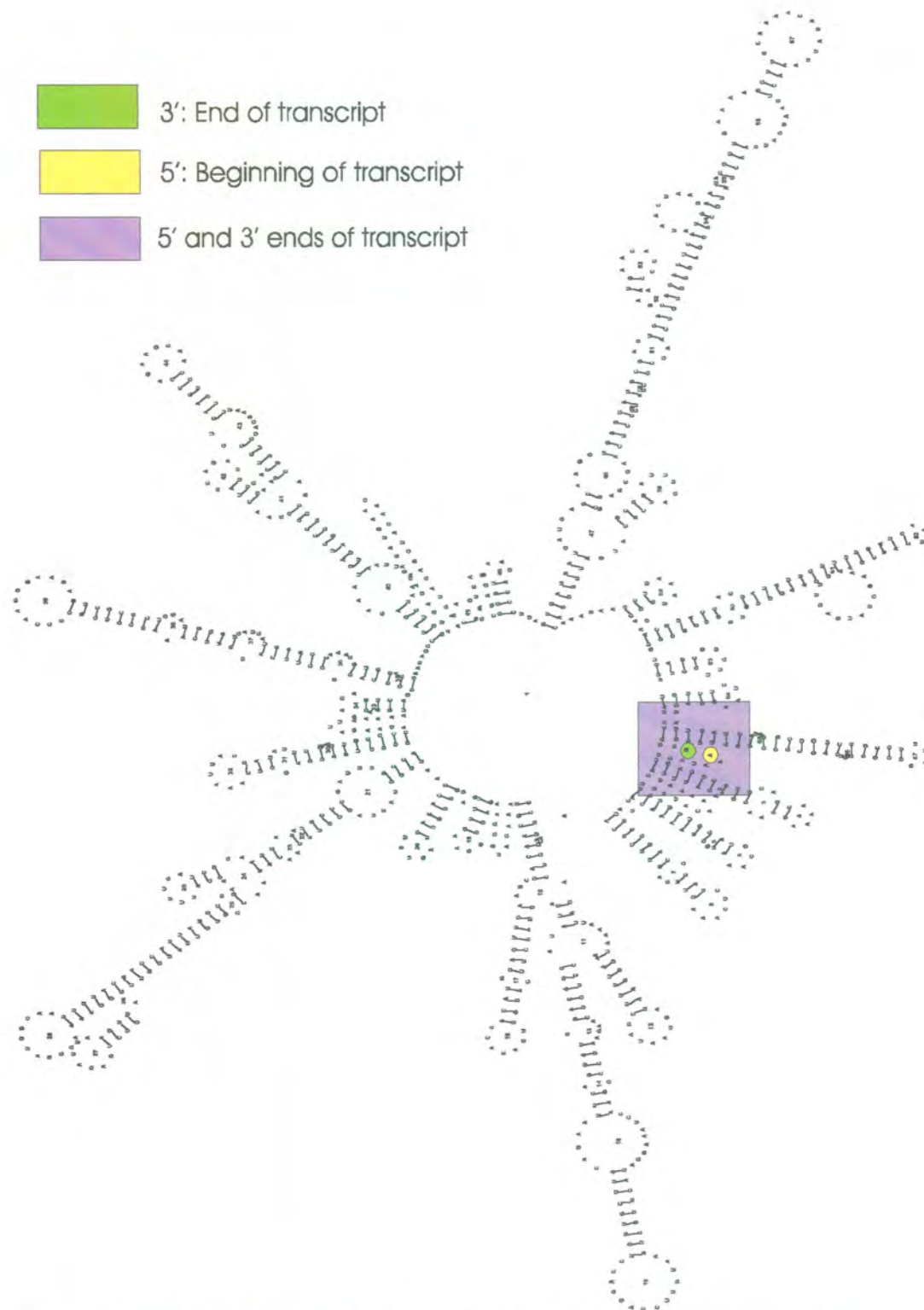


Fig. 4.13: RNA secondary structure prediction of the mutant transcript i3'BTVNS2 from which the 3' end inverted repeat sequence was removed.

4.3.3 Secondary structure analysis of different orbivirus segment 8 mRNA transcripts and identification of conserved secondary structure motifs

Secondary structure analysis of different orbivirus segment 8 mRNAs was performed in order to determine whether these sequences could also base-pair through the inverted repeat sequences and to compare the secondary structure between different members of the orbivirus genus. The results indicated that base-pairing between the 5' and 3' inverted repeat sequences does occur (Fig. 4.14 – Fig. 4.16) and yielded structural motifs that differ greatly between the different orbiviral RNA segments.

The ALIDOT program used in this analysis first generated a sequence alignment of the different orbivirus segment 8 RNAs (Fig. 4.14). Secondary structure of four different orbivirus segment 8 RNAs (EHV, AHSV, BTV and KHSV) was compared with each other by generating a consensus sequence. Sixty-six stem-loop structures were identified in BTV segment 8 mRNA. Eleven stem-loop structures were found to be conserved among the various different segment 8 orbivirus mRNAs (Table 4.2). All of the conserved structures formed part of either the 5' end (basepair 1- 490) or the 3' end (basepair 779-1124), and no conserved structures were identified in the internal part (basepair 491- 778) of the RNA segments. Thus, despite differences in the nucleotide sequence of the different orbivirus segment 8 RNAs, these sequences share conserved secondary structures. These structures may play a role in non-specific RNA-protein binding which would allow for selection of viral RNA from a pool of cellular and viral RNA.

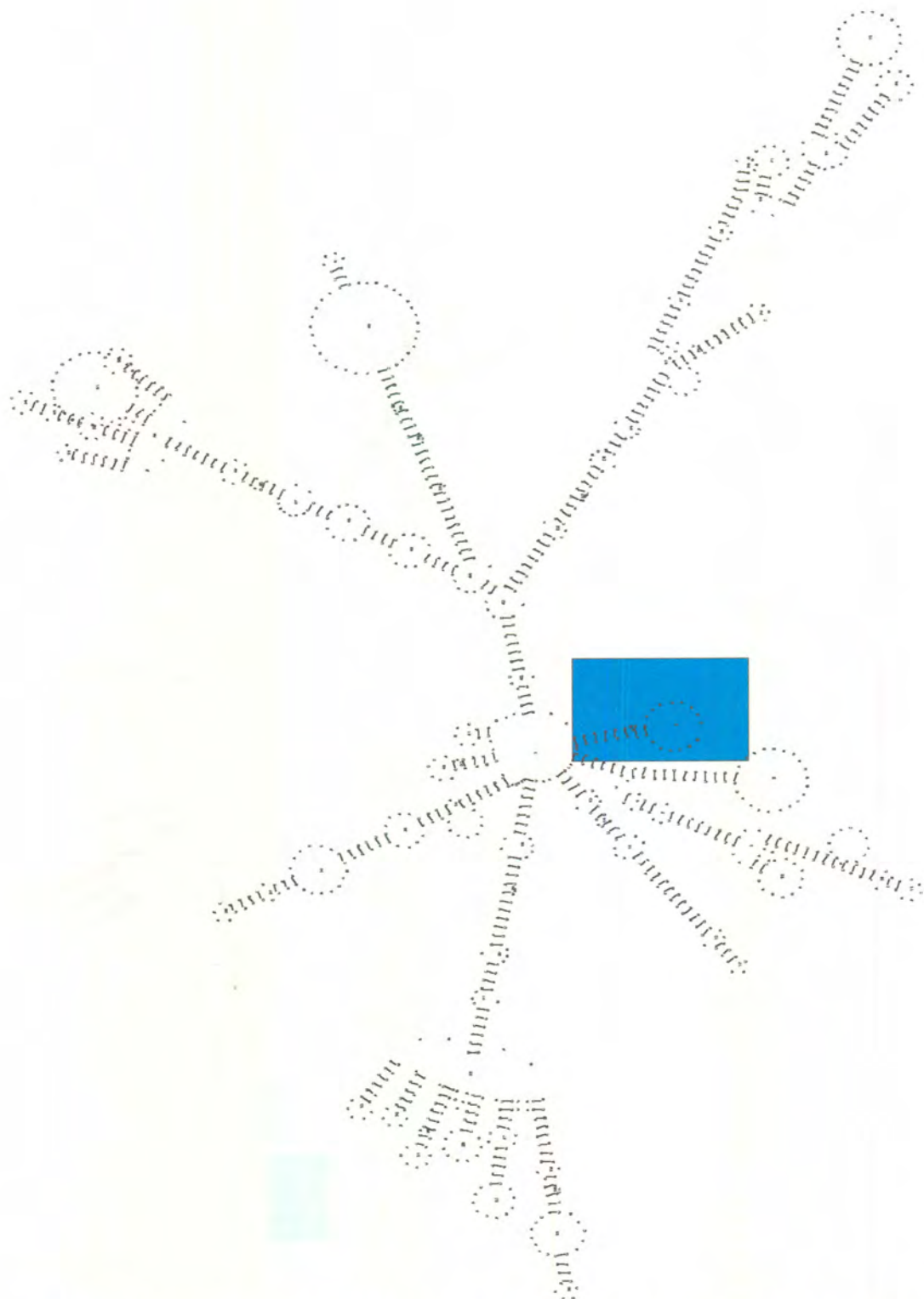


Fig. 4.14: Secondary structure analyses of the full-length EHDV segment 8 RNA indicating base-pairing at the 5' and 3' inverted repeat sequences.

Fig. 4.15: Secondary structure analyses of the full-length AHSV segment 8 RNA indicating base-pairing at the 5' and 3' inverted repeat sequences.

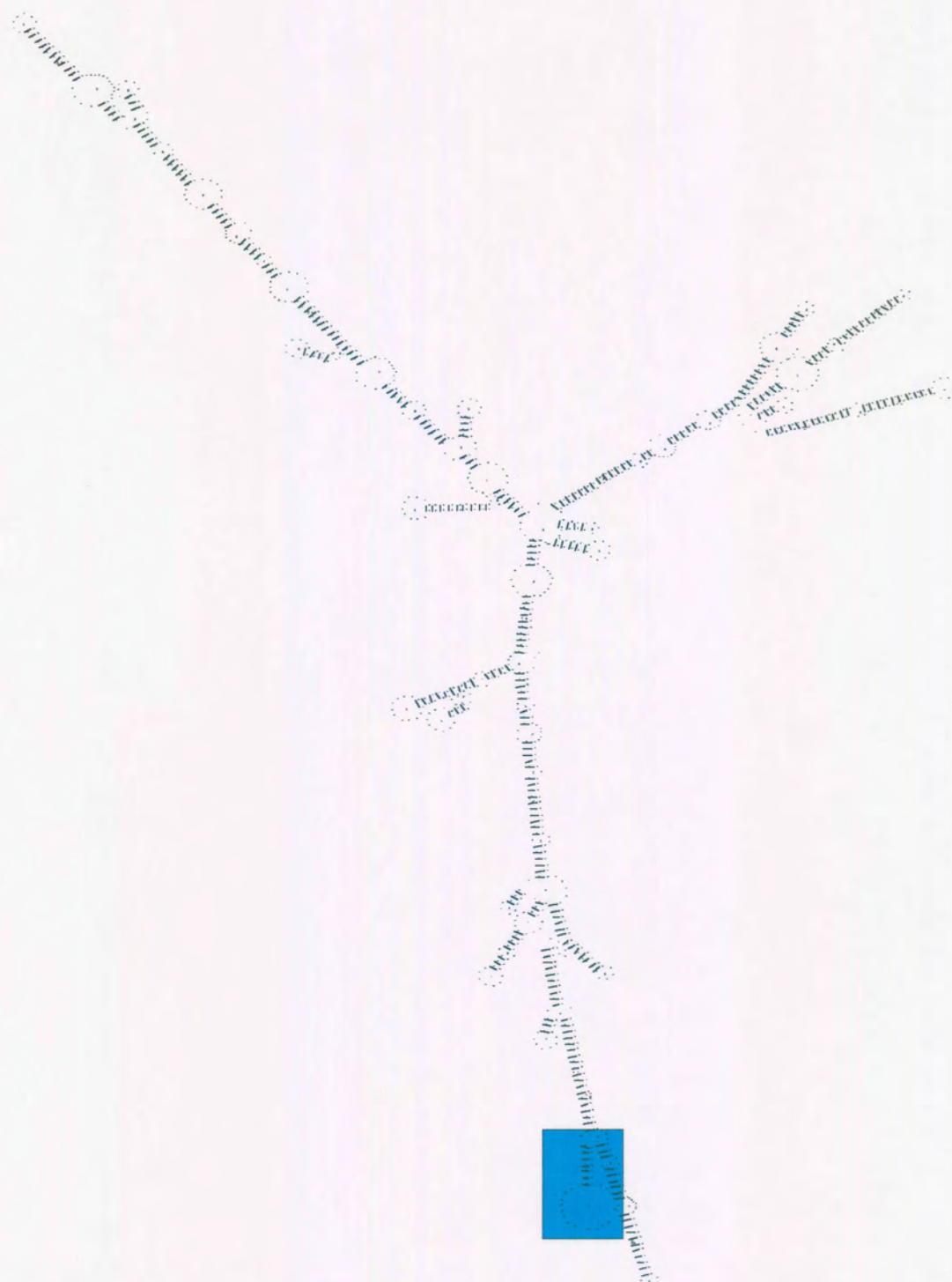


Fig. 4.16: Secondary structure analyses of the full-length KASV segment 8 RNA indicating base-pairing at the 5' and 3' inverted repeat sequences

EHDVNS2 GTTAAAAAATTCCTTGTCGAAT-----GGAGCAAAAGCAGGAGGATTCCACGAGAAG
BTVNS210 GTTAAAAAATCCTTGAGTCAT-----GGAGCAAAAGCAACGTAGATTACTAAAAA
AHSVNS2 GTTTAAAAAATCCGTTTCGTATCATGCGCAGAGGTGAGAAAGCAACAATTACGCGATC
KASVNS2 GTAAAAAATTTCTT--GACATCATGGG---TGACAGAAAGC-----GTTATACGAAAAC
* * * * *

EHDVNS2 CGTTTTTGTCTTGTATCAAAGCGGAAAAACAATATGCGGACAAATAGCAGCGAGACAATC
BTVNS210 CATTTTTGTCTTTGGATGCAAATGC TAAACACTATGCGGCAGGATCGCAAAGTTGAGTTC
AHSVNS2 AGTTTGTGTCTTGTATTTAGGACAAAAGACTTATGCGGTAAAGTGGTTAGAGCAGTAGA
KASVNS2 TGTTTGTGTCTATGATCCGAGAAGGCCACTTTTTGTTCAGAGGCGCAAATGCAGGTGA
* * * * *

EHDVNS2 ATTGCCATATTGTCAAATCAAATAGGGAGGAACCTTCGCTTTGAGGGCGGTTGCGACTCC
BTVNS210 GCAACCGTACTGTCAGATTAATAATTGGAAGAGTAATAGCTTTTAAACCTGTCAAGAATCC
AHSVNS2 TGGAGTGTATTACACCATTAAAATTGGGAGAACCGTACAATGTGGGGTACGCCAACCC
KASVNS2 GAAT---TATTATCAGGTTAAGATTGGTAGAACGCCAAATTACGAGGACAAACGCGCC
* * * * *

EHDVNS2 AGAACCGAAGGGATATGTGTTGGAATCTGTGATGTTGGCGCATATCGAATT CAGGATGG
BTVNS210 GGAACCTAAAGGATACGTGCTAAATGTTCCAGGACCTGGTGCGTACAGAATT CAGGATGG
AHSVNS2 GATTCCAAAAGTTATGTTTTGGAGATTTCGTGAATGCGGCAGCTTACCGTATTCAAGATGG
KASVNS2 GAACCCAAAGGCATATGTGGTCGAGATT CAGAGACCATGTTTCAGTCCGACTTTTAGATGG
* * * * *

EHDVNS2 AAATGATGTCATTAGTTTGATGATTTTCGGCGGATGGGGTAGAGGGTACGCAAGAACGATG
BTVNS210 ACAGGATATCATTAGCTTGATGTTTGACACCACATGGAGTTGAAGCGACACGGAAGATG
AHSVNS2 GACGGATGTTTAAAGCTTAATGATTACAGAGAGTGAATTTAGGTAACGCAACGAAACCGATG
KASVNS2 CAATGACATTATAAGCCTCATTATTT CAGAAGAGGGAATTGAAGTAACAACAGAACGATG
* * * * *

EHDVNS2 GGAAGAGTGGA AATTGAAAGCGATCTCATGCGTACCAATGGCCACAGTTCTAAATATAA
BTVNS210 GGAAGAGTGGAAGTTTGAGGGTGTTAGTGTGAC-ACCAATGGCTACTAGGGTACAACATA
AHSVNS2 GGAGGAGTGGAAGTTTCGAA-GCGTTAACACCAAGTCCAATGGCTGTGGCGGTGAATGTAG
KASVNS2 GGAGGAGTGGCTCTTTGAA-GCGCTAACACCGATTCCAATTGTAATTTCTCTGCATATTA
* * * * *

EHDVNS2 ACGGAGCGTTGGTAGACGC-GAAATCAAAGTGTCAAAGGGTATGGGGTAGTACCACCGT
BTVNS210 ACGGTTGATGGTTGATGCTGAGATTAAATGATTGCAAAGGGATGGGAATTGTACAACCAT
AHSVNS2 GGAGAGGCTCGTTGACACTGAGATTAAATATGTGAGAGGAAGCGGTGCGGTTCCACCTT
KASVNS2 AAGGTCAAAAACAGAAAGCGGAAATCAATATTGCAAAGCGAGCGGGTTATCGCACCTT
* * * * *

EHDVNS2 ACACGAGGAATGATTTTGATAGGAGAGACTTCCTGAGTTACCCGGGTCCAGAGATCAA
BTVNS210 T-ATGCGGAACGATTTTGATCGGAATGAGATGCCCGATTTGCCAGGTTGATGAGGTCAA
AHSVNS2 ACACGAAAAATGGAATGGATCGAAGAGCGATGCCCTCTTTACCGAGATAACAACCTTGG
KASVNS2 ATACGCCAAATGAGGTGATCGAAGAATTACACCGCATTCACCGGGAATAACGTTTACTG
* * * * *

EHDVNS2 AATACGATATTACAGAGCTACGACAAAAAATCAGAGAAGAAGCGGAGAAAGGTGCGGTTG
BTVNS210 ATTACGATATTGCGGAATTGCGCCAGAGATTAAAAATGAACGAGAGTCAGC-GCC---
AHSVNS2 ATGTTGGAGTTAGAGATTGCGTTTTAAAGATGAGGGAGAACAGGGAGGCAGAG-----
KASVNS2 ATGTAAGTGTCCTGTATTTTAGACAAAAAATGAGAGAAGAGCGAGAAAAAG-----
* * * * *

EHDVNS2 AACAGCCTCATAAACAGCGTTCAAACCTGAACGTGGCATGAAC-AGACCAGATT CAGAT
BTVNS210 -ACGGCTTCAG-----GTTCAAAGC-----GTGGCGCCAAAG-GGAAGAGTCGCGCTG
AHSVNS2 -AAGGGAGAAGAT-----GGAACGAGCCCTGAGTGGTGGGCTCGATATGGGAAGCTGTA
KASVNS2 -----A-----AAAGAAC-----GTACTCCAATG-ATAACACCAATTAAG
* * * * *

EHDVNS2 GAAGACCAGAACCCAGCTGGCGGCGTAGTAAATGATTGGACGTGCGAGACTCAAAAACGC
BTVNS210 GATG-----GA--TGATGATGAAGCAAAGG--TGGACGAA-GAGGCTAGAGAAAT-
AHSVNS2 GAATG-----TA--TGGGGGAGGAAGAAATGA-TGTGCGCGAGATCACCTTGATGA
KASVNS2 CCAA-----AAACG----G-AGGCG-----CCGGTGATG-
* * * * *

EHDVNS2 GATCAGGAGGCTGAACGGCGTGAGGCGCTGGAGATTAGATTGGCGGACAAACGACAGAGAA
BTVNS210 GATCCCGGAAC TAGCAGATTGGAGA----AGCTGCTGAAGCGAGAAAGCAACGTTTTT
AHSVNS2 GG-CCGGACCATCACGTACGCCGCGA----AACTTTCTGTT CAGAGCAATGAAAGTCGT
KASVNS2 GGTCCGAGCACTTCAAC-----A-----ATATCAGAAAGAAGAAATCAATAGCTGT
* * * * *

```

EHDVNS2      TCGAAGCATTCAAATTGGATAGTGAGAGGAAGAGGGAAGATCTTGAAAGAAGTCAAGAAC
BTVNS210     AAGGAGGTGGCAGCTGGGAT--TAATTGGAATCTGGATGA---GAAAGA--TGAAGAAG
AHSVNS2      TCAGATGATGTGGCAGCAAGA--CATGCTGAGTTGGTGGAGATGGAGCGA--TTAAGAAT
KASVNS2      TGGAAAGATT----TCTGAT--TATGTTAAAAAAGTTAAACTTGAATCTAATGTAGAAC
              *          *          *          *          *          *          *          *          *          *
              *          *          *          *          *          *          *          *          *          *

EHDVNS2      AGCTTC--AGGA-CGCAAG-AGGTGGAGCGCAAGAAATCCGATATGGGTGAG-GTTATAA
BTVNS210     ATGGAG--ATGAACGAGAGGATGAGGAGCGGGTGAAGACCC---TGAGTGAT-G--AT--
AHSVNS2      AATGAAGAATGAACCAGTACGTACTGAGAGTATGTGGTGTC---AAGTGATAGTGAT--
KASVNS2      -----GTGATTCAGAT--GACGAAGATGAACATGGCTCA-----AATAGCGAT--
              **          **          *          *          *          *          *          *          *          *

EHDVNS2      TTGAAGAAGACGATGAGGATT-CGGAAGAGGAGGAAGG-AGCAAGAGCTTCATATATTAC
BTVNS210     --GATGAGCAAGGTGAGGATG-CGAGTGACGATGAAC--ACCCAAAGACTCATATA--AC
AHSVNS2      --GATCAATCTGATGAGGATCAGCAGGTTGGGGGTACAGAGCCAGAAAAATTATATC--AC
KASVNS2      --TCAGATTTTGATGGTGAAC-CTAAGGATGAGG-----G-----TTACATT--AC
              *          *          *          *          *          *          *          *          *          *

EHDVNS2      TTCAGCTTATATTGAGCGCATCAGCAGGATACGCAAGA-TCAAAGATGAGCGGCTATCGA
BTVNS210     TAAGGAATACGTTGAAAAGGTT-GCAAAACAGATTAACCTGAAGGATGAACGATTCATGA
AHSVNS2      TGAGGAATATACCGTAGGCTCAACGAGGTAAGACGAAATATTCAAAGA-ATTATCTT
KASVNS2      CCAGAATTATATGAATATGATT---GAAACGGTGAAAA-TGAAAGTACCGGGCGTCGCTG
              **          *          *          *          *          *          *          *          *          *

EHDVNS2      TGTTAGCGAGCATGATGCCTCAGCAATCAGGAGAGTATACTACCACATTATT--TATTAA
BTVNS210     GTTTGTCAAGCGCTATGCCTCA--AGCGAGCGGCGGATTTGATCGCATGATTGTTACAAA
AHSVNS2      CATTGGCGATGAGAGTTCCAAAG-AATGAGGGCAATTGTGGAAACC-GATTTTTTCTAA
KASVNS2      GATTGACTCTTAAATTCGAA--AGCAGCGGGAAAACTGATGGTATGATCGCCGCTAA
              **          *          *          *          *          *          *          *          *          *

EHDVNS2      GAAACAAAAATGGGATAATGTGCCATTGTATTTAATCGATGAAATGCAAAAGAAGTATGA
BTVNS210     GAAGCTTAAATGGCAGAACGTACCACATATATTGTTTGTGATGAATCATCGAAAAGGTATGA
AHSVNS2      AAAGTGTAATGGGAGAATGTTCCGATCTACAATTACGATGAGGCTAGCGGAACTATCG
KASVNS2      AAAATGTAAATGGATAAGCGTTCCACTATTTAATATTGATAAGGATGGGTTAAGTTATGA
              **          *          *          *          *          *          *          *          *          *

EHDVNS2      GTTGCAGAGCGTCGGGAGCTGCGAACGCGTTGCTTTTGTGTT--CCAAAGGCACGAACCTA
BTVNS210     GTTGCAATGTGTGGGTGCGTGTGAGCGTGTGCTTTTGTCT--CTAAGGACATGAGCTTG
AHSVNS2      TTTTGTGTGAGTGGGAAGT--CGTACGCATTACCACTGCTGTGCTAATGACTTGAGTTAC
KASVNS2      TTTCGTAGCTGTGGGGAT-GCAAACGGCTTT-TTGTGCCTACGTGATGGTCTGAGCTAC
              **          *          *          *          *          *          *          *          *          *

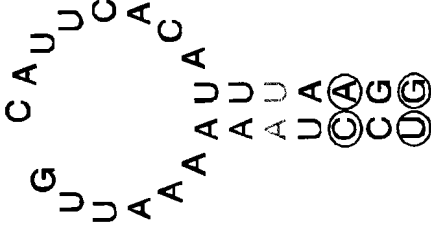
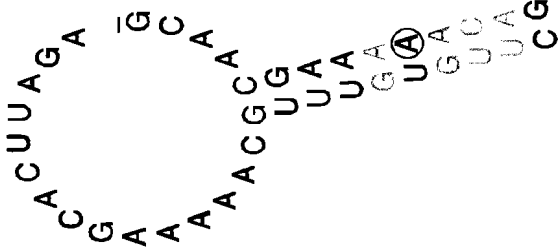
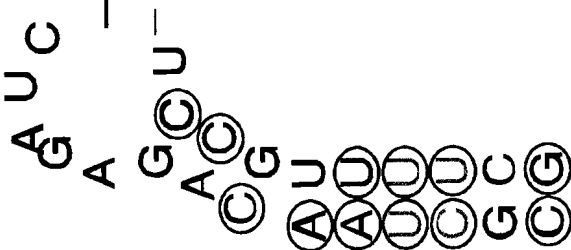
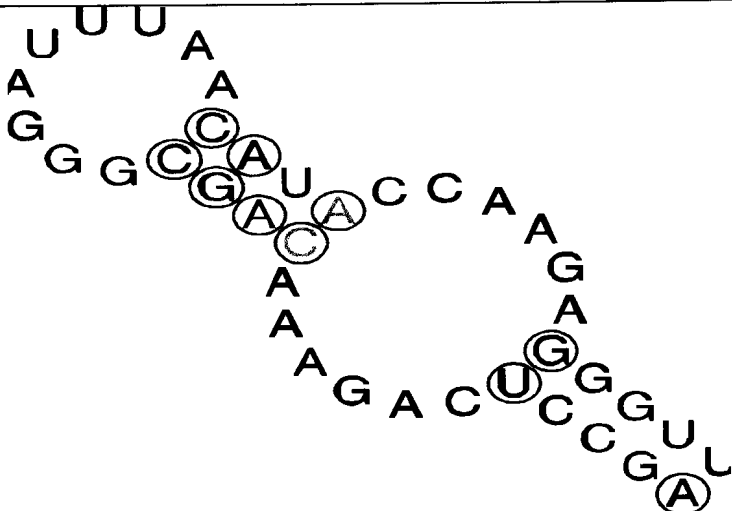
EHDVNS2      ATCATTTTACC-----GGTAGCGTCTAATCCACGCTGATGTAGCCGC-TGATGGGGGA
BTVNS210     ATCATATTGCG-----GTCGGCGTTTAGGC---GCT--TGTGACCGCGTGTTAGGGG
AHSVNS2      ATGATTTCTCCAGCAGGGGGGAGCGGTTGATCCTCCTGGGTGTACGTGGCGCATGTAACC
KASVNS2      CTAGTTCTTCCA-----GTCAGTGGATCTGTC-CACTAGTCGACTGATAGGTAATCACA
              *          *          *          *          *          *          *          *          *          *

EHDVNS2      AGGGATTTTACACTTAC
BTVNS210     GGGATTTTACACTTAC
AHSVNS2      GCGGATTTCAACATAC
KASVNS2      AGGAATAAACACTTAC
              *          *          *          *          *          *          *          *          *          *

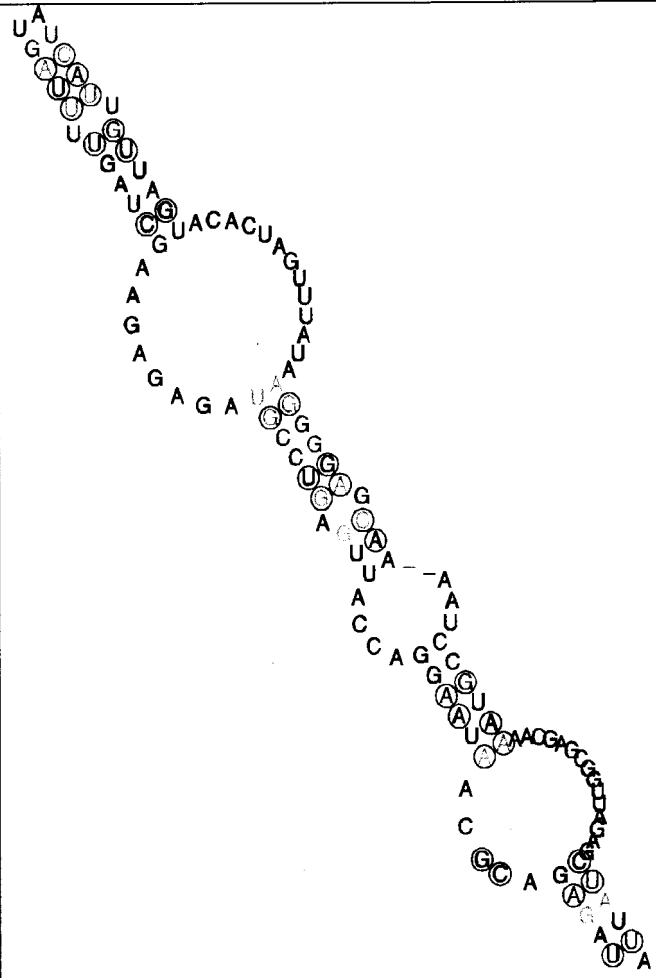
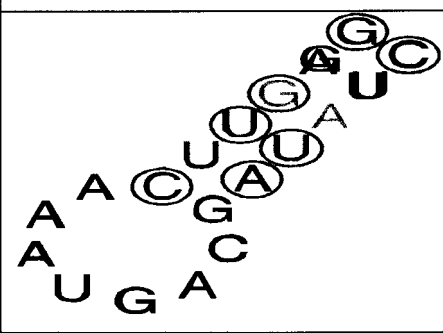
```

Fig 4.17: Multiple sequence alignment of different orbivirus segment 8 RNAs, indicating conserved sequences.

Table 4.2: Predicted consensus secondary structures for the different orbivirus segment 8 RNAs (AHSV, EHDV, BTV and KASV)

Position in nucleotide sequence of BTV NS2	Secondary structure
Basepair 1- 12 Basepair 1109 - 1124	
Basepair 23-63	
Basepair 972-996	
Basepair 136 - 175	

Basepair 202 - 227	
Basepair 230-242	
Basepair 300-323	
Basepair 891-905	
Basepair 779-793	

Basepair 420- 468 Basepair 481- 490 Basepair 812-880	
Basepair 481-490	

4.3.4 Identification of unique secondary structures on BTV-10 segment 8 RNA

After the secondary structure analysis of different orbivirus segment 8 mRNAs and identification of conserved secondary structures present in different orbivirus segment 8 RNAs, these structures were visually compared to the secondary structure of BTV-10 segment 8 RNA. Five stem-loop structures unique to BTV segment 8 RNA were identified (Fig 4.18). These structures may play a role in the specific encapsidation of segment 8 transcripts into a precursor viral particle. However, to study the importance of these structures in RNA-protein binding, further mutational experiments should be performed.

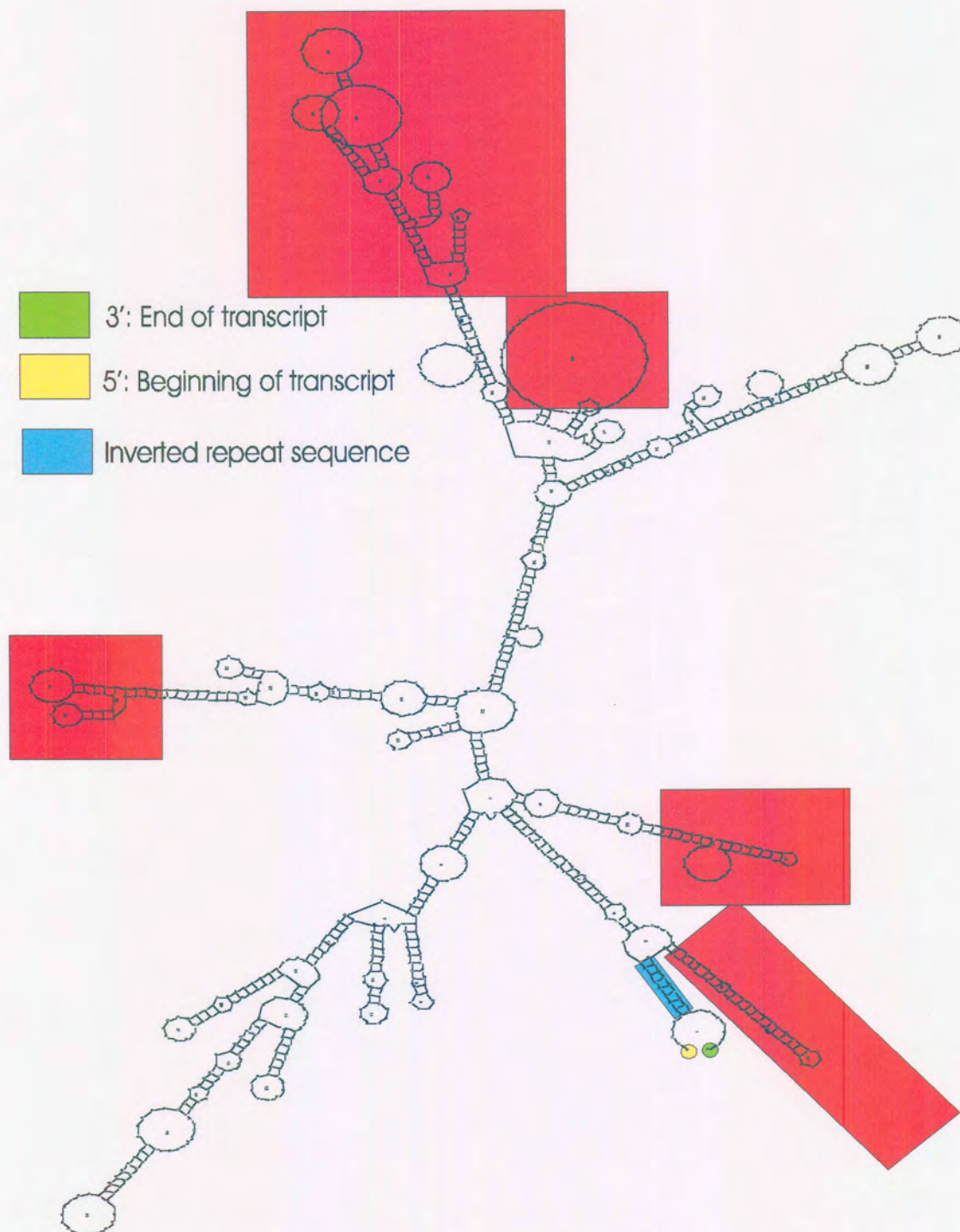


Fig 4.18: Unique secondary structures present on BTV-10 segment 8 RNA.

4.4 DISCUSSION

In this part of the study, the secondary structure of the ten different mRNA segments of BTV-10 was determined. The inverted repeat sequences present at the 5' and 3' ends of all ten segments displayed base-pairing with each other. This results in a unique secondary structure for each of the mRNA segments. Base-pairing of terminal inverted repeat sequences in RNA genome transcripts are common to most viruses with multi-segmented RNA genomes and also occurs in influenza virus (Stoeckle *et al.*, 1987), WTV (Anzola *et al.*, 1987), rice stripe virus (Takahashi *et al.*, 1990), EHDV and AHSV. Cowley *et al.* (1992) have previously predicted the secondary structures of all ten BTV-10 RNA segments, but internal parts of the genomes were deleted to generate 1200 bp transcripts that fell within the processing limits of the programme used (FOLD). Their results indicated that all the segments displayed base-pairing at the terminal inverted repeat sequences, with the exception of BTV RNA segments 4,6 and 10 for which the conserved hexanucleotide sequences present at the 5' and 3' terminal ends of the genome was also incorporated in this base-pairing. In this study, full-length RNA transcripts of BTV-10 was analyzed using RNADraw and base-pairing at the terminal inverted repeat sequences for all ten genome segments was indicated. In contrast to the results of Cowley *et al.* (1992), the conserved hexanucleotide sequences did not form part of this base-paired structure. Features common to each segment, present at the 5' or 3' end adjacent to the inverted repeat sequences, were identified in all ten BTV mRNA segments. It is possible that they mediate RNA-protein binding in some way, but the functional role of these structures may be determined by future mutational analyses. In secondary structure analysis of WTV (Xu *et al.*, 1989) 1 or 2 stem-loop structures consisting of 8 to 134 nucleotides upstream of the 3' inverted repeat was also identified.

To determine the importance of the 5' and 3' inverted repeat sequences in maintaining the secondary structure of the RNA segments, structural modelling was used to compare the higher order structures present in mutant forms of the RNA with each other and with those present in the full-length RNA. Analysis of mutant RNA transcripts lacking the 5' and/or 3' inverted repeat sequence revealed a secondary structure that was very different from that of the full-length BTV segment 8 transcript. The 5' and 3' ends of the RNA transcripts did not display base-pairing, leading to the formation of different stem-loop structures. This altered secondary structure may lead to sequences that could be required for RNA-protein binding being unavailable and consequently leads to a decline

in RNA binding. The different deletion mutants were analyzed in Chapter 2 for their binding activity and results indicated that there is a decline in RNA-protein binding.

The secondary structure of different orbivirus segment 8 RNAs was also predicted and base-pairing of the 5' and 3' inverted repeat sequences could be indicated. Eleven conserved secondary structure motifs, which were present at either the 5' or 3' terminal region of the RNA transcripts, could be identified. Dall *et al.* (1990) also indicated that the signals required for the packaging of WTV RNA transcripts are in the terminal domains of the individual segments. Even with as much as 78 % of the internal portion of the segment removed packaging still occurred. Functionally important secondary structures are likely to be conserved and knowledge of their location can be used to guide future deletion studies to determine their importance. Unique secondary structures on BTV-10 mRNA segment 8 was also identified and these may play a role in specific selection of different viral RNA segments.

In some cases proteins recognize RNA in a sequence-specific manner and in other cases it depends on the presence of specific RNA secondary structures. Prediction of the rotavirus mRNA secondary structures indicated the presence of one or more highly conserved stable stem-loop structures located near the 5' or 3' terminal end of the panhandles, but their importance in packaging has not yet been determined. Analysis of mutant turnip Crinkle virus RNA led to the identification of a 186 nucleotide region at the 3' end with a bulged hairpin loop of 28 nucleotides responsible for specific packaging of the viral RNA (Qu and Morris, 1997). Furthermore, the *Saccharomyces cerevisiae* dsRNA virus, ScVL1, recognizes viral mRNA sequences consisting of a stem with a bulged A residue topped by a loop of several bases (Yao *et al.*, 1997). It may thus be that specific stem-loop structures mediate the packaging and assortment of the orbiviral mRNAs. It is, however, important to note that computational methods merely provide structural models that may be verified or rejected by experimental methods. Thus, more detailed studies are required to determine the importance of the structures identified in this study for RNA-protein binding interactions.

CHAPTER 5: CONCLUDING REMARKS

During the replication cycle of BTV, the prototype virus of the orbivirus genus, no free pools of double-stranded RNA are observed in the cytoplasm. This has led to the assumption that the viral mRNA is first packaged whereafter the negative strands are synthesized in a precursor viral particle, using the positive sense RNAs as template. Thus, mechanisms whereby the virus can distinguish between viral and cellular RNA (non-specific binding) as well as between the ten different mRNA segments (specific binding) must exist. In order for the virus particle to be infectious, it must contain one of each of the ten different genomic RNA segments. Single-stranded binding proteins might be involved in this selection process. In BTV, three proteins (VP6, VP3 and NS2), have been reported that are capable of binding to single-stranded RNA. VP6 binds single and double-stranded RNA and is believed to play a role in the unwinding of double-stranded DNA prior to initiation of transcription and replication. VP3 also binds both dsRNA and ssRNA. NS2 is the only BTV protein that binds exclusively to ssRNA. It has therefore been postulated that NS2 is closely involved in packaging of the ssRNA molecules. The aim of this study was to identify conserved motifs on the viral mRNA that may be responsible for specific binding to the NS2 protein. Analysis of the viral RNA sequences, indicated the existence of conserved hexanucleotide sequences at the 5' and 3' ends of the genomes, but earlier studies have indicated that deletion of these sequences does not influence RNA-NS2 binding.

The first objective of this study was to identify sequences or motifs on the BTV RNA segments that may be involved in the selection of ten different RNA segments into precursor viral particles. In addition to the conserved hexanucleotide sequences, each genomic RNA segment contains inverted repeat sequences that differ in sequence and length. The importance of these sequences in BTV NS2-RNA binding was investigated. Deletion mutants were successfully constructed using PCR methodology, whereby the 5' and/or 3' inverted repeat sequences were removed from a DNA copy of BTV RNA segment 8. These mutant genes were cloned into a transcription vector under control of the T7 RNA polymerase promoter and radiolabelled RNA transcripts were synthesized *in vitro*. The NS2 protein was expressed in a eukaryotic expression system and NS2-RNA binding was investigated using a nitrocellulose-binding assay. The results obtained indicated that the inverted repeat sequences do not influence the binding directly, but it may have an indirect influence by altering the secondary structure of the mRNA and

consequently, the binding sites may no longer be available for binding by the NS2 protein.

The next objective was to investigate the use of an IAsys biosensor for its suitability in determining the binding efficiency of different orbivirus segment 8 RNAs (AHSV, EHDV and BTV) to the BTV NS2 protein. Although the previously used nitrocellulose-binding assay yielded meaningful results, it did, however, lead to the generation of radioactive waste and is also very labour-intensive. Therefore, it was necessary to investigate new approaches towards studying RNA-protein interactions. In this study, the use of an IAsys biosensor was investigated. DNA copies of AHSV and EHDV segment 8 were cloned into a transcription vector under control of the T7 RNA polymerase promoter. For these experiments, the BTV NS2 protein was expressed in the pGEX prokaryotic expression system. Although the proteins were successfully immobilized on the CMD matrix, no RNA-binding to the NS2-GST fusion protein could be obtained. The IAsys system is a rapid method that can yield you qualitative and quantitative results without labelling the RNA or purifying the GST fusion protein. Further investigations are necessary to refine the methodology for use in RNA-protein binding studies.

The selective packaging mechanism of orbiviruses is still not understood. Since the viral mRNA is packaged followed by synthesis of the dsRNA in the virus core particles, the packaging signals may be present on the viral mRNA. Computer-based predictions regarding the secondary structure of the viral mRNA segments were performed. Base-pairing could be indicated at the terminal inverted repeat sequences of all ten mRNA segments of BTV-10 as well as for other orbiviruses including AHSV, EHDV and KASV. The secondary structures of mutant transcripts lacking the inverted repeat sequences as constructed in this study, indicated a disruption of the structure when these sequences were deleted. Thus, the inverted repeat sequences may not be directly involved in binding to NS2, but they may be responsible for maintaining a specific secondary structure important for RNA-NS2 binding. Conserved secondary structures on the segment 8 mRNA of different orbiviruses that may have a functional importance was identified. Future mutagenesis experiments whereby the influence of secondary the structures in RNA-protein binding can be investigated, should be performed.

REFERENCES

Antczak, J.B. Chumelo, R., Pickup, D.J. and Joklik, W.K. 1982. Sequences at both termini of the 10 genes of Reovirus Serotype 3. *Virol.* **121**: 307-319.

Anzola, V.A., Zhengkai, X., Asamizu, T. and Nuss, D.L. 1987. Segment-specific inverted repeats found adjacent to conserved terminal sequences in wound tumor virus genome and defective interfering RNAs. *Proc. Natl. Acad. Sci. USA.* **84**: 8301-8305.

Borden, E.C., Shope, R.E. and Murphy, F.A. 1971. Physicochemical and morphological relationships of some arthropod-borne viruses to bluetongue virus – a new taxonomic group. *Physiochemical and serological studies. J. Gen. Virol.* **13**: 261-271.

Bowne, J.G. and Ritchie, A.E. 1970. Some morphological features of bluetongue virus. *Virol.* **40**: 903- 911.

Boyle, J.F. and Holmes, K.V. 1986. RNA binding proteins of bovine rotavirus. *J. Virol.* **51**: 561-568.

Boyle, J.S. and Lew, A.M. 1995. An inexpensive alternative to glassmilk for DNA purification. *TIG.* **11**: 8.

Brookes, S.M., Hyatt, A.D. and Eaton, B.T. 1993. Characterization of virus inclusion bodies in bluetongue virus-infected cells. *J. Gen. Virol.* **74**: 525-530.

Brottier, P., Nandi, P., Bremont, M. and Cohen, J. 1992. Bovine rotavirus segment 5 protein expressed in the baculovirus system interacts with zinc and RNA. *J. Gen. Virol.* **73**: 1931-1938.

Brucculeri, R. and Heinrich, G. 1988. An improved algorithm for nucleic acid secondary structure display. *Comp. Applic. Biosci.* **4**: 167-173.

Costas, J., Sutton, G., Ramadevi, N. and Roy, P. 1998. Guanylyl-transferase and RNA 5'-triphosphatase activities of the purified expressed VP4 protein of bluetongue virus: identification of the active nucleotide-binding site. *J. Mol. Biol.* **280**: 859-866.

Cowley, J.A., Walker, P.J. and Gorman B.M. 1992. Recognition sites in assembly of bluetongue viruses. *In: Bluetongue, African Horse sickness and Related Orbiviruses* (T.E. Walton and B.I. Osburn, Eds), Proceedings of the Second International Symposium, pp. 423-432. CRC Press, FL.

Dall, D.J., Anzola, J.V., Xu, Z. and Nuss, D.L. 1990. Structure specific binding of wound tumor virus transcripts by a host factor: Involvement of both terminal nucleotide domains. *Viol.* **179**: 599-608.

Eaton, B.T., Hyatt, A.D. and Brookes, S.M. 1990. The replication of bluetongue virus. *Curr. Top. Microbiol. Immunol.* **162**: 89-118.

Eaton, B.T., Hyatt, A.D. and White, J.R. 1988. Localization of the nonstructural protein NS1 in bluetongue virus-infected cells and its presence in virus particles. *Viol.* **163**: 527-537.

Enfors, S.O. 1992. Control of in vivo proteolysis in the production of recombinant proteins. *Trends Biotechnol.* **10**: 310-315.

Erasmus, B.J. 1975. Bluetongue in sheep and goats. *Aust. Vet. J.* **51**: 165-170.

Fraenkel-Conrat, H., Kimball, P. C. and Levy, J. A. General Virology, Virology 2nd Ed. pp. 1-38. Prentice-Hall, New Jersey.

Freier, S.M., Kiezek, R., Jaeger, J.A., Sugimoto N., Caruthers, M.H., Neilson, T. and Turner, D.H. 1986. Improved free-energy parameters for predictions of RNA duplex stability. *Proc. Natl. Acad. Sci. USA.* **83**: 9373 – 9377.

French, T.J., Inumara, S. and Roy, P. 1989. Expression of two related nonstructural proteins of bluetongue virus (BTV) type 10 in insect cells by a recombinant baculovirus: production of polyclonal ascitic fluid and characterization of the gene product in BTV-infected BHK cells. *J. Virol.* **63**: 3270-3278.

Fukusho, A., Yu, Y., Yamaguchi, S. and Roy, P. 1989. Completion of the sequence of bluetongue virus serotype 10 by the characterization of a structural protein, VP6, and a non-structural protein, NS2. *J. Gen. Virol.* **70**: 1677-1689.

Gambles, R.M. 1949. Bluetongue virus of sheep in Cyprus. *J. Comp. Pathol.* **59**: 176-190.

Gorman, B.M. 1983. On the evolution of Orbiviruses. *Interviol.* **20**: 169-180.

Gorman, B.M. 1992. An overview of the orbiviruses. *In: Bluetongue, African Horse sickness and Related Orbiviruses* (T.E. Walton and B.I. Osburn, Eds.), Proceedings of the Second International Symposium, pp. 335-347. CRC Press, FL.

Gorman, B.M., Taylor, J., Walker, P.J., Davidson, W.L. and Brown, F. 1981. Comparison of bluetongue type 20 with certain viruses of the bluetongue and Eubenberg serological groups of orbiviruses. *J. Gen. Virol.* **57**: 251-261.

Gorman, B.M., Taylor, J. and Walker, P.J. 1983. Orbiviruses. *In: Reoviridae* (W.K. Joklik, Ed.), pp 287-357. Plenum, New York.

Gouet, P., Diprose, J.M., Grimes, J.M., Malby, R., Burroughs, J.N., Zientara, S., Stuart, D.I., and Mertens, P.P.C. 1999. The highly ordered double-stranded RNA genome of bluetongue virus revealed by crystallography. *Cell* **97**: 481-490.

Grimes, J., Basak, A.K., Roy, P. and Stuart, D.I. 1995. The crystal structure of bluetongue virus VP7. *Nature* **373**: 167-170.

Grimes, J.M., Burroughs, J.N., Gouet, P., Diprose, J.M., Malby, R., Zientara, S., Mertens, P.P.C. and Stuart, D.I. 1998. The atomic structure of the bluetongue virus core. *Nature* **395**: 470-478.

Grimes, J., Jakana, J., Ghosh, M., Basak, A.K., Roy, P., Chiu, W., Stuart, D.I. and Prasad, B.V.V. 1997. An atomic model of the outer layer of the bluetongue virus core derived from X-ray crystallography and electron cryo-microscopy. *Structure* **5**: 885-893.

Hassan, S.S. and Roy, P. 1999. Expression and functional characterization of bluetongue virus VP2 protein: role in cell entry. *J. Virol.* **73**: 9832-9842.

Hassan, S.H., Wirblich, C., Forzan, M. and Roy, P. 2001. Expression and functional characterization of bluetongue virus VP5 protein: role in cellular permeabilization. *J. Virol.* **75**: 8356-8367.

Hatada, E., Saito, S., Okishio, N. and Fukuda, R. 1997. Binding of the influenza virus NS1 protein to model genome RNAs. *J. Gen. Virol.* **78**: 1095-1063.

Hatada, E., Tikizawa, T. and Fukuda, R. 1992. Specific binding of influenza A virus NS1 protein to the virus minus-sense RNA *in vitro*. *J. Gen. Virol.* **73**: 17-25.

He, L., Kierzek, R., Santa Lucia, J., Walter, A.E. and Turner, D.H. 1991. Nearest-neighbour parameters for G multiplied by U mismatches: 5'GU3'/3'UG5' is destabilizing in the contexts CGUG/GUGC, UGUA/AUGU, and AGUU/UUGA but stabilizing in GGUC/CUGG. *Biochemistry* **30**: 11124-11132.

Heery, D.M., Gannon, F. and Powell, R. 1990. A simple method for subcloning DNA fragments from gel slices. *TIG* **6**: 173.

Hofacker, I.L., Fekete, M., Flamm, C., Huynen, M.A., Rauscher, S., Stolorz, P.E. and Stadler, P.F. 1998. Automatic detection of conserved RNA structure elements in complete RNA virus genomes. *Nucleic Acids Res.* **26**: 3825 – 3836.

Hofacker, I.L., Fontana, W., Stadler, P.F., Bonhoeffer, L.S. Tacker, M. and Schuster, P. 1994. Fast folding and comparison of RNA secondary structures. *Chem. Monthly* **125**: 167-188.

Hofacker, I.L. and Stadler, P.F. 1999. Automatic detection of conserved base-pairing patterns in RNA virus genomes. *Comp. & Chem.* **23**: 401-414.

Hoff, G.L. and Hoff, D.M. 1976. Bluetongue and epizootic hemorrhagic disease: A review of these diseases in nondomestic artiodactyls. *J. Zoo. Anim. Med.* **7**: 26.

Howell, P.G. 1960. A preliminary antigenic classification of strains of bluetongue virus. Onderstepoort J. Vet. Res. **28**: 357.

Howell, P.G. 1963. Bluetongue. *In*: Emerging Diseases of Animals. FAO Agricultural studies No 61. pp. 111-153. Food and Agricultural Organization of the United Nations, Rome.

Howell, P.G. 1970. The antigenic classification and distribution of naturally occurring strains of bluetongue virus. J. S. Afr. Vet. Med. Assoc. **41**: 215-223.

Howell, P.G. and Verwoerd, D.W. 1971. Bluetongue virus. *In*: Virology Monographs (S. Gard, C. Hallauer and K.F. Meyers, Eds.), pp. 35-74. Springer-Verlag, New York.

Hua, J., Mansell, E.A. and Patton, J. 1994. Comparative analysis of the rotavirus NS53 gene: conservation of basic cysteine-rich region is essential for virus-specific RNA binding. J. Virol. **68**: 3990-4000.

Huismans, H. 1979. Protein synthesis in bluetongue virus-infected cells. Virol. **92**: 385-396.

Huismans, H. 1985. The use of recombinant DNA technology for the development of a bluetongue virus subunit vaccine. Onderstepoort J. Vet. Res. **52**: 149-151.

Huismans, H. and Basson, H.M. 1983. The characterization of a phosphorylated nonstructural polypeptide of BTV with affinity for RNA, pp. 173-181. *In*: Double-stranded RNA viruses (R.W. Compans and D.H.L. Bishop, Eds), pp. 173-181. Elsevier, New York.

Huismans, H. and Els, H.J. 1983. Characterization of the tubules associated with the replication of three different orbiviruses. Virol. **92**: 397-406.

Huismans, H. and Erasmus, B.J. 1981. Identification of the serotype-specific and group-specific antigens of bluetongue virus. Onderstepoort J. Vet. Res. **48**: 51-58.

Huismans, H. and Joklik, W.K. 1976. Reovirus-coded polypeptides in infected cells: Isolation of two native monomeric polypeptides with affinity for single-stranded and double-stranded RNA, respectively. *Viol.* **70**: 411-424.

Huismans, H., Van der Walt, N.T., Cloete, M. and Erasmus, B.J. 1987b. Isolation of a capsid protein of bluetongue virus that induces a protective immune response in sheep. *Viol.* **157**: 172-179.

Huismans, H. and Van Dijk, A.A. 1990. Bluetongue virus structural components. *Current topics in Microbiology and Immunology* **162**: 21-41.

Huismans, H., Van Dijk, A.A. and Bauskin, A.R. 1987. *In vitro* phosphorylation and purification of a nonstructural protein of Bluetongue virus with affinity for single-stranded RNA. *J. Virol.* **61**: 3589-3595.

Hutcheon, D. 1902. Malarial catarrhal fever of sheep. *Vet. Research* **14**: 629-633.

Hyatt, A.D., Brookes, S.M., Gould, A.R. and Eaton, B.T. 1992. Morphogenesis of bluetongue viruses: development of a model for the site of virus synthesis, translocation, and release from infected tissue culture cells, pp. 358-365. *In: Bluetongue, African Horse Sickness and Related Orbiviruses* (T.E. Walton and B.I. Osburn, Eds.), Proceedings of the Second International Symposium, pp. 358-365. CRC Press. Florida.

Hyatt, A.D. and Eaton, B.T. 1988. Ultrastructural distribution of the major capsid proteins within bluetongue virus and infected cells. *J. Gen. Virol.* **69**: 805-815.

Hyatt, A.D., Eaton, B.T. and Brookes, S.M. 1989. The release of Bluetongue virus from infected cells and their super infection by progeny virus. *Viol.* **173**: 21-34.

Hyatt, A.D., Gould, A.R., Coupar, B. and Eaton, B.T. 1991. Localization of the non-structural protein NS3 in bluetongue virus infected cells. *J. Gen. Virol.* **72**: 2263-2267.

Jacobson, S.J., Konings, D.A.M. and Sarnow, P. 1993. Biochemical and genetic evidence for a pseudoknot structure at the 3' terminus of the poliovirus RNA genome and its role in viral RNA amplification. *J. Virol.* **67**: 2961-2971.

Jaeger, J.J., Turner, D. and Zuker, M. 1989. Improved predictions of secondary structures for RNA. *Biochemistry* **86**: 7706-7710.

Kahlon, J., Sugiyama, K. and Roy, P. 1983. Molecular basis of bluetongue neutralisation. *J. Virol.* **48**: 627 –632.

Kattoura, M., Clapp, L.L. and Patton, J.T. 1992. The rotavirus non-structural protein, NS35, is a non-specific RNA-binding protein. *Virol.* **191**: 698-708.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.

LeBlois, H., French, T., Mertens, P.P., Burroughs, J.N. and Roy, P. 1992. The expressed VP4 protein of bluetongue virus binds GTP and is the candidate guanylyl transferase of the virus. *Virol.* **189**: 757-761.

Lecatsas, G. 1968. Electron microscopic study of the formation of bluetongue virus. *Onderstepoort J. Vet. Res.* **35**: 139-150.

Leipoldt, E.A. and Munro, H.N. 1988. Cytoplasmic protein binds in vitro to a highly conserved sequence in the 5' untranslated region of ferritin heavy- and light subunit-mRNAs. *Proc. Natl. Acad. Sci. USA* **85**: 2171-2175.

Libikova, H. and Casals, J. 1971. Serological characterization of Eurasian Kemerovo group viruses I. Cross complement fixation tests. *Acta Virol.* **15**: 65-78.

Loudon, P.T. and Roy, P. 1991. Assembly of five bluetongue virus proteins expressed by recombinant baculoviruses: inclusion of the largest protein VP1 in the core and virus-like particles. *Virol.* **180**: 798-802.

Loudon, P.T. and Roy, P. 1992. Interaction of nucleic acids with core-like and subcore-like particles of bluetongue virus. *Viol.* **191**: 231 – 236.

Lustig, A. and Levine, A. J. 1992. One hundred years of virology. *J. Virol.* **66**: 4629-4631.

Maree, F.F. and Huismans, H. 1997. Characterization of tubular structures composed of nonstructural protein NS1 of African horse sickness virus expressed in insect cells. *J. Gen. Virol.* **78**: 1077-1082.

Martin, S.A. and Zweerink, H.J. 1972. Isolation and characterization of two types of bluetongue virus particles. *Viol.* **50**: 495-506.

Mason, J.H., Coles, J.D.W.A. and Alexander, R.A. 1940. Cultivation of bluetongue virus in fertile eggs produced on a vitamin – deficient diet. *Nature* **145**: 1022-1023.

Mattion, N.M., Cohen, J., Aponte, C. and Estes, M.K. 1992. Characterization of an oligomerization domain and RNA-binding properties on rotavirus nonstructural protein NS34. *Viol.* **190**: 68-83.

Matzura, O. and Wennborg, A. 1996. RNAdraw: an integrated program for RNA secondary structure calculation and analysis under 32-bit Microsoft Windows. *CABIOS* **12**: 247-249.

McCaskill, J.S. 1990. The equilibrium partition function and base pair binding probabilities for RNA secondary structure. *Biopolymers* **29**: 1105-1119.

Mertens, P.C. and Bamford, D.H. The RNAs and Proteins of dsRNA Viruses. 2 Junie 1998. 30 Nov 2001. <http://www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/Orbivirus.htm>

Mertens, P.P.C., Brown, F. and Sanger, D.V. 1984. Assignment of the genome segments of bluetongue virus type 1 to the proteins that they encode. *Viol.* **135**: 207-219.

Mertens, P.P.C., Burroughs, J.N. and Anderson, J. 1987. Purification and properties of virus particles, infectious subviral particles, and cores of bluetongue virus serotypes 1 and 4. *Virol.* **157**: 375-38.

Mertens, P.P.C., Pedley, S., Cowley, J., Burroughs, J.N., Corteyn, A.H., Jeggo, M.H., Jennings, D.M. and Gorman, B.M. 1989. Analysis of the roles of bluetongue virus outer capsid proteins VP2 and VP5 in determination of virus serotype. *Virol.* **170**: 561-565.

Mertens, P.P.C. and Sangar, D.V. 1985. Analysis of the terminal sequences of the genome segments of four orbiviruses. *Virol.* **140**: 55-67.

Murphy, F.A., Borden, E.C., Shope, R.E. and Harrison, A. 1971. Physicochemical and morphological relationships of some arthropod-borne viruses to bluetongue virus - A new taxonomic group. Electron microscopic studies. *J. Gen. Virol.* **13**: 273-288.

Murphy, F.A., Fauquet, C.M., Bishop, D.H.L., Ghabril, S.A., Jarvis, A., Marteli, G.P., Mayo, M.A. and Summers, M.D. 1995. Virus taxonomy: the classification and nomenclature of viruses. *Arch Virol. suppl.* **10**: 592 .

Neitz, W.O. 1948. Immunological studies on bluetongue in sheep. *Onderstepoort J. Vet. Res.* **23**: 93-136.

Nel, L.H., Picard, L.A. and Huismans, H. 1990. A characterization of the nonstructural protein from which the virus-specified tubules in epizootic hemorrhagic disease virus infected cells is composed. *Virus Res.* **18**: 219-230.

Patton, J.T. 1995. Structure and function of the rotavirus RNA-binding proteins. *J. Gen. Virol.* **76**: 2633-2644.

Poncet, D., Laurent, S. and Cohen, J. 1994. Four nucleotides are the minimal requirement for RNA recognition by rotavirus non-structural protein NSP3. *EMBO J.* **13**: 4165-4173.

Possee, R.D. and Howard, S.C. 1987. Analysis of the polyhedrin gene promoter of the *Autographa californica* nuclear polyhedrosis virus. *Nucleic Acids Res.* **15**: 10233-10248.

Qu, F. and Morris, T.J. 1997. Encapsidation of turnip crinkle virus is defined by a specific packaging signal and RNA size. *J. Virol.* **71**: 1428-1435.

Ramadevi, N., Burroughs, N.J., Mertens, P.P.C., Jones, I.M. and Roy, P. 1998. Capping and methylation of mRNA by purified recombinant VP4 protein of bluetongue virus. *Proc. Nat. Acad. Sci.* **95**: 13537-13542.

Ramadevi, N. and Roy, P. 1998b. Bluetongue virus core protein VP4 has nucleoside triphosphate phosphohydrolase activity. *J. Gen. Virol.* **79**: 2475-2480.

Rao, C.D., Kiuchi, A. and Roy, P. 1983. Homologous terminal sequences of the genome double-stranded RNAs of Bluetongue virus. *J. Virol.* **46**: 378-383.

Roy, P. 1989. Bluetongue virus genetics and genome structure. *Virus Res.* **13**: 179-206.

Roy, P., Adachi, A., Urakawa, T., Booth, T.F. and Thomas, C.P. 1990a. Identification of bluetongue virus VP6 protein as a nucleic acid binding protein and the localization of VP6 in virus infected vertebrate cells. *J. Virol.* **64**: 1-8.

Roy, P., Marshall, J.J.A. and French, T.J. 1990b. Structure of the Bluetongue virus genome and its encoded proteins. *Current Topics in Microbiology and Immunology* **162**: 43 –87.

Rubio, I., Buckle, P., Trutnau, H. and Wetzker, R. 1997. Real-time assay of the interaction of a GST fusion protein with a protein ligate using resonant mirror technique. *BioTechniques* **22**: 269-271.

Samal, S.K., Livingston, C.W., McConnell, S. and Ramig, R.F. 1987. Analysis of mixed infection of sheep with bluetongue virus serotypes 10 and 17: evidence for genetic reassortment in the vertebrate host. *J. Virol.* **61**: 1086-1091.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular cloning, a laboratory manual. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Stäuber, N., Martinez-Costas, J., Sutton, G., Monastyrskaya, K. and Roy, P. 1997. Bluetongue virus VP6 protein binds ATP and exhibits an RNA-dependent ATPase function and a helicase activity that catalyzes the unwinding of double-stranded RNA substrates. *J. Virol.* **71**: 7220-7226.

Stoeckle, M.Y., Shaw, M.W. and Chopin, P.W. 1987. Segment-specific and common nucleotide sequences in the noncoding regions of influenza B virus genome RNAs. *Proc. Nat. Acad. Sci.* **84**: 2703-2707.

Stoltz, M.A., Van der Merwe, C.F., Coetzee, J. and Huismans, H. 1996. Subcellular localization of the nonstructural protein NS3 of African horsesickness virus. *Onderstepoort J. Vet. Res.* **63**: 57-61

Studdert, M.J., Panborn, J. and Addison, R.B. 1966. Bluetongue virus structure *Virol.* **29**: 509-511.

Sugiyama, K., Bishop, D.H.L. and Roy, P. 1981. Analyses of the genome of bluetongue viruses recovered in the United States. *Virol.* **114**: 210-217.

Summers, M.D. and Smith, G.E. 1987. A manual of methods for Baculovirus vectors and insect cell culture procedures. Texas Agric. Exp. Station Bull. No 1555.

Takahashi, M., Toriyama, S., Kikuchi, Y., Hayakawa, T. and Ishihama, A. 1990. Complementarity between the 5'- and 3'-terminal sequences of rice stripe virus RNAs. *J. Gen. Virol.* **71**: 2817-2821.

Tan, B., Nason, E., Staeuber, N., Jiang, W., Monastyrskaya, K. and Roy, P. 2001. RGD tripeptide of bluetongue virus VP7 protein is responsible for core attachment to *Culicoides* cells. *J. Virol.* **75**: 3937-3947.

Taraporewala, Z.F., Chen, D. and Patton, J.T. 2001. Multimers of the bluetongue virus nonstructural protein, NS2, possess nucleotidyl phosphatase activity: similarities between NS2 and Rotavirus NSP2. *Viol.* **280**: 221-231.

Theron, J., Uitenweerde, J.M., Huismans, H., and Nel, L.H. 1994. Comparison of the expression and phosphorylation of the non-structural protein NS2 of three different orbiviruses: evidence for the involvement of ubiquitous cellular kinase. *J. Gen. Virol.* **75**: 3401-3411.

Theron, J., Huismans, H. and Nel, L.H. 1996. Identification of a short domain within the non-structural protein NS2 of epizootic haemorrhagic disease virus that is important for single strand RNA-binding activity. *J. Gen. Virol.* **77**: 129-137.

Theron, J. and Nel, L.H. 1997. Stable protein-RNA interaction involves the terminal domains of bluetongue virus mRNA, but not the terminally conserved sequences. *Viol.* **229**: 134-142.

Thomas, C.P., Booth, T.F. and Roy, P. 1990. Synthesis of bluetongue virus encoded phosphoprotein and formation of inclusion bodies by recombinant baculovirus in insect cells: it binds the single-stranded RNA species. *J. Gen. Virol.* **71**: 2073-2083.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**: 4673-4680.

Turner, D., Sugimoto, N. and Freier, S. 1988. RNA structure predictions. *Ann. Rev. Biophys. Chem.* **17**: 167-192.

Uitenweerde, J.M., Theron, J., Stoltz, M.A. and Huismans, H. 1995. The multimeric nonstructural NS2 protein of bluetongue virus, African horsesickness virus, and Epizootic haemorrhagic disease virus differ in their single-stranded RNA-binding ability. *Viol.* **209**: 624-632.

Urakawa, T. and Roy, P. 1988. Bluetongue virus tubules made in insect cells by recombinant baculoviruses: Expression of the NS1 gene of bluetongue virus serotype 10. *J. Virol.* **62**: 3919-3927.

Urakawa, T., Ritter, D.G. and Roy, P. 1989. Expression of largest RNA segment and synthesis of VP1 protein of bluetongue virus in insect cells by recombinant baculovirus: association of VP1 protein with RNA polymerase activity. *Nucleic Acids Res.* **17**: 7395-7402.

Van Regenmortel, C.M., Fauquet, D.H.L., Bishop, E.B., Carstens, M.K., Estes, S.M., Lemon, J., Maniloff, M.A. Mayo, D.J. McGeoch, C.R. Pringle, and R.B. Wickner 2000. *Virus Taxonomy: The Classification and Nomenclature of Viruses. The Seventh Report of the International Committee on Taxonomy of Viruses.* pp 1167. Academic Press, San Diego.

Van Staden, V. and Huismans, H. 1991. A comparison of the genes which encode non-structural protein NS3 of different orbiviruses. *J. Gen. Virol.* **72**: 1037-1044.

Van Staden, V., Theron, J., Greyling, B.J., Huismans, H. and Nel, L.H. 1991. A comparison of the nucleotide sequences of cognate NS2 genes of three different orbiviruses. *Virol.* **185**: 500-504.

Verwoerd, D.W. 1969. Purification and characterization of bluetongue virus. *Virol.* **38**: 203-212.

Verwoerd, D.W., Els, H.J., De Villiers, E.M. and Huismans, H. 1972. Structure of the bluetongue virus capsid. *J. Virol.* **10**: 783-794.

Verwoerd, D.W. and Huismans, H. 1972. Studies on the *in vitro* and *in vivo* transcription of the bluetongue virus genome. *Onderstepoort J. Vet. Res.* **39**: 186-192.

Verwoerd, D.W., Huismans, H. and Erasmus, B.J. 1979. Orbiviruses. *In: Comprehensive Virology* (H. Fraenkel-Conrat and R.R. Wagner, Eds.), Vol. 14 pp. 285-345. Plenum Press, New York.

Verwoerd, D.W., Louw, H. and Oellermann, R.A. 1970. Characterization of Bluetongue virus ribonucleic acid. *J. Virol.* **5**: 1-7.

Wu, X., Chen, S., Iwata, H., Compans, R.W. and Roy, P. 1992. Multiple glycoproteins synthesized by the smallest RNA segment (S10) of bluetongue virus. *J. Virol.* **66**: 7104-7112.

Xu, G., Wilson, W., Mecham, J., Murphy, K., Zhou, E. and Tabachnick, W. 1997. VP7: an attachment protein of bluetongue virus for cellular receptors in *Culicoides variipennis*. *J. Gen. Virol.* **78**: 1617-1623.

Yao, W., Adelman, K. and Bruenn, J.A. 1997. *In vitro* selection of packaging sites in a double-stranded RNA virus. *J. Virol.* **71**: 2157-2162.

Yi, C.K., Bansal, O.B., Lui, H.M., Chatterjee, S. and Roy, P. 1996. Sequences within the VP6 molecule of bluetongue virus that determine cytoplasmic and nuclear targeting of the protein. *J. Virol.* **70**: 4778-4782.

Zuker, M. and Stiegler, P. 1981. Optimal computer folding of larger RNA sequences using thermodynamics and auxiliary information. *Nucl. Acids Res.* **9**: 133-148.

COMMUNICATIONS

Presenter's name: W. Markotter

Co-Authors: Theron J and Nel. L.H.

Title: The importance of the terminal inverted repeat sequences within bluetongue virus mRNA for interaction with the virus nonstructural protein NS2

Name of conference:

Seventeenth Congress of the South African Genetics Society, 27 – 30 June 2000. South Africa. Pretoria

Type of Presentation:

Poster

Presenter's name: W. Markotter

Co-Authors: Theron J and Nel. L.H.

Title: The importance of conserved motifs within bluetongue virus mRNA for interaction with the virus nonstructural protein NS2

Name of conference:

BioY2K Combined Millennium Meeting, Grahamstown, South Africa, January 2000

Type of Presentation:

Poster

Presenter's name: W. Markotter

Co-Authors: Theron J and Nel. L.H.

Title: Binding determinants in the interaction of the Orbivirus nonstructural protein, NS2 with single-stranded RNA

Name of conference:

XIth International Congress of Virology, Sydney, Australia, 9-13 August 1999

Type of Presentation:

Poster