The characterization of inner core protein VP6 of African Horsesickness Virus

By

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The light shines in the darkness, and the darkness has never put it out.

John 1:5

dedicated to my husband David
and my parents Jimmy and Jean

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SUMMARY

The characterization of inner core protein VP6 of African Horsesickness Virus

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For the degree PhD

VP6 is one of the minor structural core proteins of African horsesickness virus. The minor core proteins VP1, VP4 and VP6 are presumed to constitute the dsRNA dependent RNA polymerase transcription complex of the virus. In the *Orbivirus* prototype bluetongue virus (BTV), VP6 has a helicase activity. The aim of this investigation was to characterize the primary structure and nucleic acid binding function of the inner core protein VP6 of African horsesickness virus (AHSV).

To characterize the primary structure of AHSV VP6, VP6 genes of serotypes 3 and 6 were cloned and sequenced. Both genes encode a 369 amino acid polypeptide.

A comparison to the VP6 proteins of other *Orbiviruses* indicated that in all cases the proteins are rich in basic residues and in glycine. The proteins are highly conserved within serogroups but the conservation between serogroups is low. VP6 of AHSV-3 and AHSV-6 have 93.5% identity and 96% similarity in amino acid residues. AHSV-6 VP6 has 27% identical and 46% similar amino acid residues to BTV-10 VP6. Phylogenetic analysis of four orbivirus VP6 genes indicated that AHSV and BTV are most closely related to each other. Motifs characteristic of known helicases were identified by sequence analysis. Glycine rich protein motifs and a N-glycosylation signal were present. No nucleic acid binding motifs identified in other proteins were found in AHSV VP6.

To characterize the VP6 protein of AHSV VP6, the genes were expressed using both a baculovirus and a bacterial expression system. Proteins were found to be soluble and the VP6 expressed in insect cells was found to be N-glycosylated.

The nucleic acid binding function of AHSV VP6 was investigated. Bacterially expressed VP6 was demonstrated to bind nucleic acids by electrophoretic mobility shift assays. Baculovirus expressed VP6 bound double and single-stranded RNA and DNA in nucleic acid overlay protein blot assays. Competition assays indicated that VP6 may have a preference for binding to RNA rather than DNA. Glycosylation was found to play no direct role in nucleic acid binding but the binding is strongly dependent on the NaCl concentration.

A series of truncated VP6 peptides were produced to investigate the importance of localized regions in nucleic acid binding. Two partially overlapping peptides were found to bind dsRNA at pH 7.0, while other peptides with the same overlap did not. Binding appeared to be influenced by charge as reflected by the isoelectric points (pI) of the peptides and experiments indicating the effect of pH on the binding activity. However, only peptides containing amino acid residues 190 to 289 showed binding activity. This region corresponded to the region on BTV VP6 that contains two binding domains. It is proposed that the dsRNA binding domain in AHSV VP6 is a sequence of positively charged amino acids constituting a domain that determines the nucleic acid binding characteristics of the peptide. The mechanism of binding of baculovirus expressed VP6 in a nucleic acid overlay protein blot is proposed to be charge related.

DECLARATION

I declare that the thesis which I hereby submit for the degree Philosophiae Doctor at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature: …… Date: ……..

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LIST OF ABBREVIATIONS

AHS	African horsesickness
AHSV	African horsesickness virus
AHSV-6	African horsesickness virus serotype 6
amp	ampicillin
AMV	Avian myeloblastosis virus
ATCC	American type culture collection
ATP	adenosine-5'-triphosphate
bp	base pairs
BRDV	Broadhaven virus
BSA	bovine serum albumin
BTV	bluetongue virus
°C	degrees Celsius
cDNA	complementary DNA
CER	chicken embryo reticulocyte
Ci	Curie
CLP	core-like particle
cpm	counts per minute
Da	Daltons
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
DEPC	diethylpyrocarbonate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
ds	double-stranded
DTT	1,4-dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
e.g.	exempli gratia (for example)
EHDV	epizootic haemorrhagic disease virus
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assays
et al.	et alia (and others)
etc.	et cetera (and so forth)
EtBr	ethidium bromide (3,8-diamino-6ethyl-5-phenylphenathridium bromide)
FCS	foetal calf serum
g	gravitational acceleration
HCV	hepatitis C virus
hr/s	hour / hours
h.p.i.	hours post infection
HPRI	human placental ribonuclease inhibitor
i.e.	it est (that is)
IPTG	isopropyl-β-D-thiogalactopyranoside
KAc	potassium acetate
kb	kilobasepairs
kDa	kilodalton
LacZ	β-galactosidase gene
LB	Luria-Bertani
M	molar
µg	microgram
µl	microlitre
mM	millimolar
mA	milliampere
mCi	millicurie
MCS	multiple cloning site
mg	milligram
MHV	mouse hepatitis virus
min	minutes
ml	millilitre
mmol	millimol
MMOH	methylmercuric hydroxide
m.o.i.	multiplicity of infection
M_r	molecular weight
mRNA	messenger ribonucleic acid
NaAc	sodium acetate
NaOH	sodium hydroxide
nm	nanometre
NS	nonstructural
OD	optical density
ORF	open reading frame
OVI	Onderstepoort Veterinary Institute

PAGE	polyacrylamide gel electrophoresis
PBS	protein buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pfu	plaque forming units
p.i.	post infection
pmol	picomol
PNK	polynucleotide kinase
PSB	protein solvent buffer
RC	replicase complex
RF	replicative form
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
SDS	sodium dodecyl sulphate
sec	seconds
Sf9	*Spodoptera frugiperda* (fall armyworm) cells
SF	super families
ss	single-stranded
SV40	simian virus 40
TCA	trichloroacetic acid
TdT	terminal deoxynucleotidyl transferase
TEMED	N,N,N',N',-tetramethylethylenediamine
tet	tetracycline hydrochloride
Tris	Tris-hydroxymethyl-aminomethane
U	units
UHQ	ultra high quality water
UV	ultraviolet
V	volts
VIB	virus inclusion bodies
VLP	virus-like particle
VP	virus protein
w/v	weight per volume
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

LIST OF BUFFERS

PBS:

137mM NaCl, 2.7 mM KCl, 4.3mM $Na_2HPO_4.7H_2O$, 14mMKH_2PO_4, pH 7.3

PSB (2x):

0.125M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol

SBB:

50mM NaCl; 1mM EDTA; 10mM Tris-HCl, pH 7; 0.02% Ficoll; 0.02% polyvinylpyrollidone; 0.02% BSA

STE buffer:

0.15M NaCl, 0.01M Tris-HCl pH 7.6, 0.001M EDTA

STE-Tx buffer:

0.15M NaCl, 0.01M Tris-HCl pH7.6, 0.001M EDTA, 0.5% Triton-X100

TAE buffer:

0.04M Tris-acetate, 0.002M EDTA, pH 8.5

TE buffer:

0.01M Tris-HCl pH 7.6, 0.001M EDTA

TGS buffer:

0.025M Tris-HCL pH 8.3, 0.192M glycine, 0.1% SDS

CHAPTER 1: LITERATURE REVIEW

1.1 INTRODUCTION

In 1898 researchers identified infectious agents that were smaller than the smallest known bacteria. Viruses are obligate intracellular parasites that use the machinery of the host cell to replicate. They consist of a DNA or RNA genome surrounded by a protein capsid, which may be surrounded by an envelope in some viruses (Fields, 1998, Gale *et al*., 2000). Virus classification rests largely on the characteristics of the viral genome and virus structure.

Numerous viruses perform virion associated enzymatic activities, which vary according to the strategy for replication of their nucleic acid. Genome transcription is an important stage in the life cycle of a virus as the product of this process must be recognized by the machinery of the host cell for the production of viral proteins necessary for genome replication and the assembly of progeny virions. In the case of viruses with dsRNA genomes, host cells do not have the endogenous enzymes for transcribing from a dsRNA template. Also, dsRNA is never released into the cell because it would evoke a cellular defence response (Bamford, 2000). As a result, dsRNA viruses produce their own enzymes to synthesize mRNA transcripts within the core particle (Lawton *et al*., 2000).

It is thought that the mechanisms involved in the production of mRNA are similar in most viruses having dsRNA genomes. Functional similarities include a RNA-dependent RNA polymerase that produces single-stranded mRNA transcripts from the dsRNA genome as well as replicating the viral genome from single-stranded RNA templates. Viruses infecting eukaryotes have a capping enzyme for synthesizing 5' capped mRNA for translation by the eukaryotic machinery (Lawton *et al*., 2000). Double-stranded RNA viruses require unwinding of the genome segments by a helicase prior to transcription (Kadaré and Haenni, 1997).

The International Committee for the Taxonomy of Viruses (ICTV) recognizes eight families of dsRNA viruses (Mertens, 2004). This includes the family Reoviridae, members of which infect mammals, other vertebrates, insects and plants. Within the Reoviridae family is the genus *Orbivirus* (Levy *et al*., 1994). Virus species within the *Orbivirus* genus that are known to infect equids include African horsesickness virus (AHSV), equine encephalosis virus (EEV) (Mertens *et al.*, 2000) and Peruvian horse sickness virus (PHSV) which was recently assigned to the genus.

African horsesickness virus causes an infectious but non-contagious disease of equines (Coetzer and Erasmus, 1994). Mortality rates in naive populations of horses can exceed 98% (Mertens *et al*., 2000). It is accordingly considered to be one of the most lethal diseases of horses and has been given Office International des Epizooties (OIE) list A

status. Although mortality of horses as a result of AHS occurs annually in South Africa, major epidemics of the disease also occur (Coetzer and Erasmus, 1994).

In BTV, the prototype virus of the *Orbivirus* genus, inner core protein VP6 has been identified as the dsRNA helicase of the virus (Stäuber *et al*., 1997). The focus of this study is an investigation of the primary structure and nucleic acid binding characteristics of the proposed helicase of African horsesickness virus (VP6). In order to provide a background to the study, the literature review will be focussed on aspects of African horsesickness virus and viral helicase activities.

1.2 FAMILY REOVIRIDAE

The viruses classified within the family Reoviridae have dsRNA genomes. The name Reovirus comes from respiratory enteric orphan virus, which is the name proposed for a group of viruses originally classified as echo virus 10 (Sabin, 1959). Gomatos and Tamm (1963) reported that these viruses have a dsRNA genome. There are 11 genera within this family (*Orthoreovirus, Orbivirus, Cypovirus, Aquareovirus, Rotavirus, Coltivirus, Fijivirus, Phytoreovirus, Seadornavirus, Mycoreovirus* and *Oryzavirus*). A new genus has been proposed for insect reoviruses as well as some unassigned viruses (Mertens, 2004). They share specific characteristics despite an enormous diversity in hosts, transmission mechanisms, geographical distribution and pathological outcomes. Members of the family *Reoviridae* share numerous structural properties. These properties include: isometric virions with a 60 – 80 nm diameter; an inner protein coat with one or two icosahedral capsids; an outer capsid which may appear incomplete or missing altogether leaving a naked core. The core consists of the inner protein coat and the genome and has transcriptase activity. The virion has a characteristic molecular weight and buoyant density in CsCl (Urbano and Urbano, 1994).

The dsRNA genome is segmented, consisting of 10 – 12 segments. Genetic recombination by means of reassortment of genome segments occurs between closely related viruses (Gorman and Taylor, 1985; Urbano and Urbano, 1994). Virus classification and taxonomy has recently been reviewed. In this review, parameters have been outlined for the identification of virus species within the genera of the family *Reoviridae* (Mertens *et al*., 2000). The capacity for reassortment of genome segments is the primary determinant of virus species (Calisher and Mertens, 1998; Mertens *et al*., 2000). In the absence of evidence of segment reassortment, serological comparisons are usually used to investigate the relatedness of virus isolates. In some cases, such as bluetongue virus and African horsesickness virus, the serogroup specific antigen, VP7, is used for these serological comparisons. There is a list of species parameters for the genus that is used to identify the members of the same or distinct virus species or genera (Mertens *et al*., 2000).

Viral replication, which occurs in the cytoplasm, involves conservative transcription of each genomic segment into full-length plus strands which are capped and methylated

by endogenous enzymes. These function as mRNAs for viral protein synthesis or templates for progeny viral RNA (Urbano and Urbano, 1994). Members of the family *Reoviridae* are clustered into genera on the basis of minor structural differences and major extrinsic properties such as host range. These genera consist of viruses isolated from terrestrial and nonterrestrial vertebrates and invertebrates as well as plants (Calisher and Mertens, 1998). A large number of varied viruses are grouped under the genus *Orbivirus* (Urbano and Urbano, 1994).

1.3 ORBIVIRUSES

The outer shell of orbiviruses has no discernible capsomeric structure, while the inner shell consists of 32 ring shaped capsomeres arranged in icosahedral symmetry (Oellermann *et al*., 1970; Gorman and Taylor, 1985; Gould and Hyatt, 1994). The name originates from the Latin word *orbis* which means a ring or circle (Gorman and Taylor, 1985; Gould and Hyatt, 1994). Although orbiviruses are not a homogeneous group, recognized serological groups contain genomes consisting of 10 dsRNA segments (Verwoerd *et al.*, 1970; Gorman and Taylor, 1985).

Orbiviruses are able to multiply in insects and in vertebrates (Gorman and Taylor, 1985). There are 20 serogroups or species of orbiviruses as well as 12 tentative species (Mertens, 2004). The classification of viruses within this genus was originally determined by serologic tests (Urbano and Urbano, 1994) and later supported by molecular characteristics (Calisher and Mertens, 1998). Orbiviruses include pathogens of man (Changuinola virus, Corriparta virus, Great Island virus and Lebombo virus); domestic and wild animals (bluetongue virus, African horsesickness virus, Equine encephalosis virus, Peruvian horse sickness virus, Epizootic haemorrhagic disease of deer virus, Orungo virus, Wongorr virus, Wad Medani virus and Palyam virus), birds (Chenuda virus, Ieri virus and Umatilla virus), bats (Chobar gorge virus) and marsupials (Wallal virus and Warrego virus) as well as Eubenangee virus and St Croix River virus with unknown vertebrate hosts (Gorman, 1979; Calisher and Mertens, 1998; Mertens *et al*., 2000). The type species of the genus is bluetongue virus (BTV). Orbiviruses are transmitted by *Culicoides* midges, ticks, phlebotomine flies and anopheline and culicine mosquitoes (Calisher and Mertens, 1998).

1.4 AHSV AND BTV EPIDEMIOLOGY, TRANSMISSION AND GEOGRAPHICAL DISTRIBUTION

Pitchford and Theiler proposed in 1903 that AHS may be transmitted by biting insects as they found that horses housed in mosquito-proof enclosures could be protected against infection (Coetzer and Erasmus, 1994). In 1944, it was demonstrated that certain species of the biting midge *Culicoides* transmit bluetongue virus. It was further proposed that horsesickness may be transmitted in the same fashion (Du Toit, 1944). AHSV and BTV cause annual outbreaks of disease in the southern hemisphere. This is

characteristic of many *Culicoides*-transmitted orbiviruses including EHDV (Mellor and Boorman, 1995).

C. imicola has been considered the most important stock associated species in summer rainfall areas of southern Africa and the only proven vector of BTV and AHSV in this subregion (Burrage and Laegreid, 1994; Venter and Meiswinkel, 1994; Meiswinkel, 1997). Various regions of South Africa have been found to be free of *C. imicola*, these include Port Elizabeth (Meiswinkel, 1997) and the eastern Orange Free State (Venter and Meiswinkel, 1994). In an outbreak of AHS in the cooler, mountainous region of South Africa between February and May of 1998, of twenty seven species of Culicoides captured in the area, AHSV was only isolated from *C. bolitinos* (Meiswinkel and Paweska, 2003). In a survey of *Culicoides* species in Portugal, although *C. imicola* was found to be the most abundant summer vector, *C. obsoletus* and *C. pulicaris* also have a wide distribution in the area. Capela *et al.* (2003) suggest a role for these species in the transmission of BTV and AHSV. BTV-9 caused outbreaks in Bosnia Herzegovina in 2002 which is further north than the range of *C. imicola* suggesting a role for *C. pulicaris* and/or *C. obsoletus* which are abundant across most of northern Europe (Takamatsu *et al.*, 2003). Temperature has been shown to play a role in infection rate and virogenesis in *Culicoides* species with a decline in replication at lower temperatures. Infectious viruses persist, however, and latent virus replication occurs in flies transferred to higher temperatures (Bradley *et al*., 1995; Wellby *et al*., 1996). The global distributions of BTV and AHSV are limited to geographical conditions where competent vectors are present and to seasons climatically favourable for vector activity (Mellor and Boorman, 1995). The disease disappears after the first frosts (Mellor 1993; Barnard, 1998). Some evidence has been presented suggesting that emergence of AHS from its enzootic zones may be due to long range flights of infected arthropod hosts (Mellor 1993). The reappearance of AHS in successive years is often caused by different serotypes of AHSV. This suggests that outbreaks are caused by new introductions of the virus and not reactivation of over wintering virus (Barnard, 1998). Changes in the global distribution of arthropod vectors of BTV and AHSV may result in an alteration in the distribution of the viruses (Mellor and Boorman, 1995). Viraemia in the mammalian hosts lasts for a maximum of one hundred days. If winter conditions last in excess of one hundred days, BTV and AHSV was not expected to survive from one year to the next. BTV and AHSV have been known to survive the winter in the absence of adult insect vectors in western Turkey, Corsica/Sardinia, Calabria (Italy), Serbia and Kosovo (approximately two years after previous reports of the disease). In these locations, *Culicoides* are absent for several months each year when severe winter conditions prevail. Takamatsu *et al*. (2003), propose that sheep $\gamma\delta$ T-cells that are continuously infected with BTV can be converted to lytic infection following interaction with skin fibroblasts or an antibody to $\gamma\delta$ T-cell-restricted surface protein WC-1. When *Culicoides* midges feed, the feeding area becomes inflamed. Large numbers of $\gamma\delta$ T-cells move to the site of inflammation. The interaction of $\gamma\delta$ T-cells that are continuously infected with BTV and skin fibroblasts would result in an increase in the number of viruses at the

feeding areas. This may increase transmission of BTV to the insect vector. Takamatsu *et al.* (2003), suggest this as a new mechanism that may account for BTV over wintering in the absence of vector insects. White *et al*. (2005), have proposed a mechanism for overwintering of BTV in their *Culicoides* hosts. They detected BTV RNA of segment 7 and to a lesser extent segment 2 RNA in larvae and pupae of *C. sonorensis.* They suggest that BTV may overwinter in the invertebrate host with possible down regulation of segment 2 expression during continuous virus infection.

BTV is distributed world wide between the latitudes 35°S and 40°N. Although bluetongue has occurred in Europe it is not endemic to the continent (Mellor and Boorman, 1995). A serological survey for BTV in Kazakhstan showed seropositive animals at the most northern limit of the BTV distribution range (Lundervold *et al.*, 2003). Vertebrate hosts for BTV include domestic and wild ruminants such as cattle, sheep and goats as well as camels, elephants and predatory carnivores (Calisher and Mertens, 1998; Mertens *et al*., 2000).

AHSV is distributed across sub-Saharan Africa with an enzootic band from Senegal and Gambia to Ethiopia and Somalia. AHS occurs in South Africa and has occurred in Egypt (Mellor, 1993; Mellor and Boorman, 1995). Although thought to be confined to Africa, outbreaks of AHSV have occurred in the Middle East, S.W. Asia, India, Cyprus and Spain. Such outbreaks are thought to be the result of increased movement of horses (Powell, 1985, Mellor and Hamblin, 2004). Low levels of antibodies against AHSV have been detected in free-living elephants. This is, however, thought to be the result of frequent exposure to infected biting midges. Significant levels of neutralizing antibodies against AHSV have not been detected in elephants and they are accordingly not regarded as a source of AHSV (Barnard *et al*., 1995). Dogs contract a highly fatal form of AHS after natural infection or ingestion of infected horse meat (Coetzer and Erasmus, 1994). They are also thought to be an unlikely reservoir for African horse sickness virus (Braverman and Chizov-Ginzburg, 1996). The most susceptible hosts for AHSV in southern Africa are horses, donkeys and zebra. The most likely candidate for a game vertebrate reservoir is zebra which are present in areas endemic for AHSV (Barnard, 1998).

1.5 ORBIVIRUS INFECTION

Bluetongue virus particles adsorb to and penetrate the cells to be infected by means of a pinocytotic vesicle (Lecatsas, 1968). From 8 h.p.i., virus specific structures such as virus inclusion bodies (VIB), virus specific tubules and virus particles may be observed in infected cells (Lecatsas, 1968; Hyatt *et al*., 1989). Virus is released from infected cells by means of budding and extrusion through the cell membrane. Released particles enter the cells by endocytosis (Hyatt *et al*., 1989). BTV is thought to multiply in the regional lymph nodes following the bite of an infected midge. From there it spreads to the rest of the body. The development of characteristic viral structures has shown that replication occurs primarily in the endothelial cells, pericytes of capillaries and small

blood vessels. Cytopathic changes in cells eventually result in hypoxia, oedema and haemorrhaging (Verwoerd and Erasmus, 1994).

In the case of AHSV, regional lymph nodes appear to be the site of primary replication. AHSV spreads throughout the animal by means of viraemia (Burrage and Laegreid, 1994). The target organs are the lungs and lymphoid tissue throughout the body (Coetzer and Erasmus, 1994). Theiler initially recognized the association of AHSV with erythrocytes (Henning, 1956). As a result of severe injury to endothelial cells, it seems that this is an important site of secondary replication. Virulence appears to be associated with the ability to cause endothelial damage (Burrage and Laegreid, 1994).

1.6 AFRICAN HORSESICKNESS PATHOGENESIS

The incubation period and severity as well as overall outcome of the disease are determined mainly by virus virulence and susceptibility of the animal. AHSV may cause severe pathological symptoms in the vertebrate host (Mertens *et al*., 2000) that can progress rapidly from first symptoms to death (Koekemoer and van Dijk, 2004). Serotype 9 is considered slightly less virulent than serotypes 1 – 8 (Coetzer and Erasmus, 1994). There are four distinct clinicopathological syndromes of AHS described by Theiler in 1921. In order of severity: fever form, subacute cardiac form (“dikkop” or “thick head” where subcutaneous swelling of the head is present), acute or mixed form and pulmonary form (“dunkop” or “thin head” where subcutaneous swelling is absent). The different forms vary in mortality between 0 and 100% (Henning, 1956; Mellor, 1993; Burrage and Laegreid, 1994; Coetzer and Erasmus, 1994). Oedematous changes and effusions into body cavities, especially the lungs, and visceral and serosal haemorrhages indicate endothelial damage and are often found in fatal cases of AHS (Coetzer and Erasmus, 1994).

1.7 DETECTION OF AHSV IN INFECTED AND VACCINATED HORSES

Methods of detection of AHSV include the use of viral antigens or nucleic acids. In the case of antigens, the use of anti-AHSV antiserum is serogroup specific. It lacks sensitivity with only 50% success in a trial (Laegreid, 1994). In another study utilizing immunological screening, the earliest serological markers corresponded mainly to VP5, VP6 and NS2 and may potentially be used as group-specific diagnostic reagents (Martinez-Torrecuadrada *et al*., 1997). RT-PCR detection based on the nucleic acid sequence of the gene encoding NS2 (segment 8) was found to be 100% successful in a trial using DNA extracted from blood or tissue. RT-PCR of the NS2 gene was also found to be serogroup specific (Laegreid, 1994; Stone-Marschat *et al.*, 1994). Single tube RT-PCR of segment 7 of AHSV has been proposed as a mechanism for diagnosis of AHSV. Using AHSV specific primers the authors were able to detect all 9 serotypes (Zientara *et al*., 1994; Zientara *et al*., 1995). Koekemoer and van Dijk (2004), developed

a rapid serotyping procedure based on a single RT-PCR of AHSV segment 2. A universal primer set allowed amplification of a short fragment at the 5' terminal of segment 2 of all 9 AHSV serotypes. DsRNA isolated from tissue or organ samples from infected horses was used to produce a serotype-specific probe for reverse line blot hybridization to identify the AHSV serotype responsible for the infection. This procedure would be extremely effective in the case of an AHS virus outbreak where a rapid response is required to control the outbreak.

Purified VP2 of AHSV may be used as a diagnostic tool to detect AHSV antibodies in field samples, serotyping and evaluation of protective antibody levels in horses (Martínez-Torrecuadrada *et al*., 1994).

Some 80 – 90% of horses vaccinated with inactivated purified AHSV did not develop antibodies against NS3 of AHSV expressed as a fusion protein in *Escherichia coli* cells. Laviada *et al*. (1995) have suggested the usefulness of NS3 in discriminating between infected and vaccinated horses.

1.8 ORBIVIRUS MORPHOLOGY

Most of the initial knowledge of the molecular biology of orbiviruses arose from research conducted on BTV. Verwoerd *et al.* (1972), found that as in the case of reovirus, seven polypeptides were present in the capsid of the bluetongue virus. Of these, 3 are minor proteins (VP1, VP4 and VP6) which are located in the inner core. The inner core is made up of two major proteins VP7 and VP3. The outer capsid consists of major proteins VP2 and VP5. There are at least 3 non-structural proteins, NS1, NS2 and NS3 (Verwoerd *et al*., 1972; Gould and Hyatt, 1994).

There are three distinct BTV particles: virions, cores and subcores. The virion is an icosahedral particle with a blurred appearance distinct from reovirus and rotavirus (Huismans and van Dijk, 1990; Roy, 1996). Removal of the outer capsid protein layer gives rise to core particles. The core particles are characterized by 32 capsomeres arranged in icosahedral symmetry with a triangulation number of T-3 (Els and Verwoerd, 1969). Immediately after infection, most of the virions are uncoated to core particles, following which a large number are converted to subcore particles during further infection (Huismans *et al*., 1987b). The subcore particle is thought to be the scaffold for the assembly of the capsomeres (VP7) (Burroughs *et al.*, 1995). According to Grimes *et al*. (1998), the core capsid is separated into two layers: a thin inner shell made up of 120 triangular plates of VP3 which is made rigid by the addition of 260 trimers of VP7 (Prasad *et al*., 1992; Stuart *et al*., 1998). The core particle is associated with an RNA-dependent RNA polymerase activity (Verwoerd and Huismans, 1972; Roy, 1992). The subcore particles consist of one major protein, three minor proteins and dsRNA (Huismans and van Dijk, 1990).

Electron microscope evidence showed the similarity between BTV and AHSV (Oellermann *et al*., 1970). Both the BTV and AHSV genomes consist of 10 dsRNA

segments (Verwoerd, 1969; Verwoerd *et al*., 1970). The segments are designated 1 to 10 in order of decreasing size on a polyacrylamide gel (Huismans and van Dijk, 1990) or L (large) 1 – 3, M (medium) 4 – 6 and S (small) 7 – 10 (Roy, 1992). Each genome segment codes for at least one polypeptide (Mertens *et al*., 1984). The coding assignments are summarized in table 1.1.

Table 1.1: Coding assignments for BTV and AHSV.

Segment	Size in base pairs		Protein [a, b]	Size in amino acids		Location [a, b]	Estimated number / particle (BTV)	Function [a]
	BTV-10 [b]	AHSV		BTV-10 [a,b]	AHSV			
1 (L1)	3954	[c]3965$_{(9)}$	VP1	1302	1253[c]	Inner core	~12[q]	RNA polymerase
2 (L2)	2926	[f]3205$_{(9)}$ [n]3221$_{(3)}$	VP2	956	1020[f] 1057[n]	Outer capsid	180[b,q]	Outer capsid protein, serotype specific antigen, cell entry
3 (L3)	2772	[d]2792$_{(6)}$	VP3	901	842[d]	Core	120[q]	Scaffold for VP7
4 (M4)	[o]2011	[h]1978$_{(3)}$	VP4	654	599[h]	Inner core	20[q]	Capping enzyme and guanylyl transferase
5 (M5)	1769[q]	[g]1748$_{(6)}$	NS1	552[m,q]	516[g]	Infected cell	NA	Nonstructural protein tubules [k,g]
6 (M6)	1638[q]	[d]1564$_{(6)}$	VP5	526[q]	518[d]	Outer capsid	360[q]	Structural protein – outer capsid, interacts with core
7 (S7)	1156	[d]1050$_{(6)}$	VP7	1156	280[d]	Core	780[b,q]	Group specific antigen
8 (S8)	1124[p]	[e]1166$_{(9)}$	NS2	357[p]	356[e]	Infected cell	NA	Binds ssRNA, found in VIBs, involved in virus replication
9 (S9)	[p]1046	[i]1169$_{(3/6)}$	VP6	328	369[i]	Inner core	60[q]	Binds ssRNA and dsRNA, NTPase and helicase
10 (S10)	822	[j]758$_{(3)}$	NS3	229	217[j]	Infected cell	NA	Virus release
			NS3A	216	207[j]		NA	

a) Huismans and van Dijk, 1990; b) Roy, 1992; c) Vreede and Huismans, 1998, d) Williams *et al*., 1998, e) van Staden *et al*., 1991, f) Venter *et al*., 2000, g) Maree and Huismans, 1997, h) Van den Bout and Huismans, unpublished data, I) Turnbull *et al*., 1996, j) van Staden and Huismans, 1991 k) Roy, 1996 l) Hall *et al*., 1989, m) Lee and Roy, 1987, n) Vreede and Huismans, 1994 o) Yu *et al*., 1987 p) Fukusho *et al*., 1989 q) Mertens, 2004.

1.9 STRUCTURE AND FUNCTION RELATIONSHIPS OF ORBIVIRUS GENES AND GENE PRODUCTS

In the last 15 years a wealth of information about the structure and function of the *Orbivirus* gene products has accumulated. The genomes of BTV and AHSV and other orbiviruses have been cloned and sequenced and the proteins expressed in baculovirus or other expression systems. High resolution X-ray crystallography has elucidated structure-function relationships of *Orbivirus* genes and gene products. The following section will highlight the most important aspects of these relationships.

1.9.1 THE OUTER CAPSID: VP2 AND VP5

Iwata *et al*. (1992) investigated the evolutionary relationships among the four major capsid proteins of African horsesickness virus, bluetongue virus and Epizootic haemorrhagic disease virus. Of the four capsid proteins, VP2 is the most variable with only 19-24% identical amino acids. As it is found on the outer capsid, it may be under immune pressure so that it evolves to accommodate host responses. Amino acid sequences of VP2 proteins in BTV vary between 40 and 70% conservation between different serotypes (Fukusho, 1987). VP2 of AHSV serotypes 3 and 4 were found to have 50.5% identity with two regions of high variability (Vreede and Huismans, 1994). Multiple alignment of the VP2 amino acid sequences for all nine serotypes of AHSV showed that homology between serotypes varied from 71.4% between AHSV-1 and AHSV-2 and 47.6% between AHSV-2 and AHSV-9 (Potgieter *et al*., 2003). VP2 contains serotype specific determinants for the virus (Huismans and Erasmus, 1981; Mecham *et al*., 1986; Roy, 1992).

In AHSV it was found that the major antigenic domain of VP2 is located in the central domain, with neither the N- half nor C-terminal regions demonstrating immunogenicity (Martínez-Torrecuadrada and Casal, 1995). Using truncated peptides of VP2 of AHSV, Venter *et al*. (2000) demonstrated the presence of a strong linear epitope in a large hydrophilic domain between amino acids 369 and 403. A filamentous phage library was used to determine sequences that may form part of a discontinuous neutralization epitope in VP2 of AHSV-3. Antigenic regions were compared with corresponding regions on three other serotypes revealing regions which may potentially be used for serological discrimination between AHSV serotypes (Bentley *et al*., 2000).

Huismans *et al*. (1987c) showed that BTV VP2 induced neutralizing antibodies in sheep which provided full protection against virulent challenge. Stone-Marschat *et al*. (1996), inoculated horses with a vaccinia construct of segment 2 cDNA. They found that immunized horses developed serum neutralizing antibodies and were clinically normal after virulent challenge. These results demonstrate that VP2 may be used alone to elicit a protective response and suggest that a subunit vaccine based on AHSV VP2 alone may be effective.

The main function of the outer capsid is cell attachment and penetration (Grimes *et al*., 1998). Hassan and Roy (1999), determined the oligomeric nature of VP2 of BTV. Their results show that most purified VP2 protein forms dimers and to a lesser extent trimers. Purified VP2 was found to have virus haemagglutinin activity. VP2 of BTV is responsible for entry into mammalian cells and may be responsible for BTV transmission by the *Culicoides* vector to vertebrate hosts (Hassan and Roy, 1999).

VP5 which is largely unexposed on the capsid surface shows 43 – 45% identical amino acids between the different orbiviruses (Iwata *et al*., 1992). Although VP5 is located on the outer capsid it does not appear to have any neutralizing activity when applied on its own. However, when used in conjunction with VP2, vaccinated sheep demonstrate higher levels of neutralizing antibodies to BTV (Roy, 1992; DeMaula *et al*., 2000). This indicates an involvement of VP5 in protection and virus neutralization. Roy (1992) proposed that VP5 may enhance the immune response as a result of interaction with VP2. O'Hara *et al.* (1998), have suggested a role for VP5 in expression of the intermediate phenotype and thus control of virulence. They also propose that VP2 which has a role in cell entry may influence virulence by affecting tissue or cell tropism. VP5 of BTV was found to bind to mammalian cells but was not involved in cell entry. VP5 induces cytotoxicity by permeabilizing mammalian and *Culicoides* insect cells (Hassan *et al*., 2001). Cytotoxicity has been localized to two amphipathic helices at the amino terminal. Another feature of VP5 of BTV that is consistent with membrane penetration activity is the formation of trimers in solution (Hassan *et al*., 2001). It seems that the function of VP5 lies in the mediation of virus-cell penetration.

Synthetic BTV VLPs consisting of VP2 and VP5 of different serotypes have been assembled using recombinant baculoviruses. These VLPs were used to produce serum with high neutralizing antibody titres against live BTV suggesting a potential use as an anti-BTV vaccine (Loudon *et al*., 1991). The same proteins expressed in *Saccharomyces cerevisiae* failed to produce a protective response to a BTV challenge as a result of conformational differences to the native proteins (Martyn *et al*., 1994). VP2 expressed in a baculovirus system has been demonstrated as a potential vaccine for AHS in horses (Roy *et al.*, 1996). However, du Plessis *et al.* (1998) have demonstrated that baculovirus expressed VP2 of AHSV is present in the form of insoluble aggregates with only 10% soluble protein. Their results further established that only soluble VP2 was capable of inducing neutralizing antibodies. The insoluble nature of VP2 is a limitation to its usefulness as a vaccine. Co-expression of VP2, VP5 and VP7 of AHSV serotype 4 has been shown to induce low levels of neutralizing antibodies and give protection against virulent virus challenge in horses (Martínez-Torrecuadrada *et al*., 1996).

Grimes *et al*. (1998) report that core particles of BTV from which the outer capsid has been removed retain infectivity for insect vectors even though their infectivity for mammalian cells has been greatly reduced.

1.9.2 THE CORE

BTV enters the cell by removal of the outer capsid and release of the core into the cytoplasm of the cell. The genome remains within the protein core which functions as a molecular engine (Grimes *et al*., 1998).

There is a large amount of conservation within the capsid genes of BTV, AHSV and EHDV (Williams *et al*., 1998). The most conserved capsid protein VP3 shows 57 – 58% amino acid identity between serogroups. The main function of VP3 appears to be structural. It provides protection for the dsRNA genome (Hwang *et al.*, 1994). Although VP7 is also very conserved it is not as highly conserved as VP3 (44 – 46% identity) (Iwata *et al*., 1992; Williams *et al*., 1998).

AHSV VP7 expressed in Sf cells self assembles to form flat, mostly hexagonal crystals such as are seen in BHK cells (Oellermann *et al.*, 1970; Chuma *et al*., 1992; Burroughs *et al*., 1994). VP7 is exceptionally hydrophobic (Roy, 1992). X-ray crystallography studies have shown that the core capsid has icosahedral symmetry. Two hundred and sixty VP7 trimers form a lattice on top of a thin shell of 120 VP3 monomers (Grimes *et al*., 1997). Thirteen icosahedrally independent copies of VP7 form the outer layer of the core capsid. They are arranged so that four trimers are in general positions and one has its molecular three-fold axis aligned with the icosahedral three-fold axis. The core has small pores at the icosahedral three-fold axes and larger pores at the five-fold axes (Grimes *et al*., 1998).

VP3 subunits display icosahedral symmetry within the subcore layer. There are two sets of VP3 subunits with a triangular wedge type appearance. The amino acids of the protein are arranged in a plate-like three-domain structure. The three domains are: the apical domain (near the 5-fold axes of the icosahedron); the carapace (forms a rigid plate) and the dimerization domain (forms a quasi-two-fold interaction). Interaction between the VP7 and VP3 layers of the core capsid occurs at flattish, mostly hydrophobic surfaces. Grimes *et al*. (1998), propose that the core may be thought of as being constructed by the crystallization of VP7 trimers onto the subcore of VP3 subunits. The driving force of this reaction may be the interaction of each trimer with the underlying VP3 layer.

Six epitopes have been mapped to the VP7 molecule of BTV. One such epitope located at the N-terminus is accessible on the surface of both intact virions and core particles (Wang *et al*., 1996a). Some evidence has been presented for the use of VP7 as a subunit vaccine for BTV (Wade-Evans *et al*., 1996) and AHSV (Wade-Evans *et al*., 1997). A series of substitutions based on the X-ray structure of VP7 were designed for BTV. These site-directed mutations targeted amino acids believed to be involved in interaction within the VP7 subunit and between subunits. The purpose being to determine the effect of certain substitutions on VP7 trimer formation and core assembly (Limn *et al*., 2000). Such information has important biological implications especially for

development of VP7 as a epitope delivery system. Similar research has been conducted on AHSV VP7 in our laboratory.

Co-expression of VP3 and VP7 of BTV and AHSV in insect cells results in the spontaneous formation of core-like particles (Hewat *et. al*., 1992; Maree *et al.*, 1998). Recombinant baculoviruses expressing VP2, VP5, VP7 and VP3 of BTV allow synthesis of non-infectious, double-shelled, virus-like particles when co-infected in insect cells. These VLPs are the same size and have the same appearance as authentic BTV virions (French *et al*., 1990).

VP7 appears to be involved in the binding of BTV vector insect membranes. Xu *et al*. (1997) suggest that VP7 may initiate specific binding to membrane receptors and facilitate VP2 and VP5 in virus cell entry. This evidence suggests a role in mediation of cell attachment and penetration for the core proteins (Grimes *et al*., 1998). Enzymatic functions of the virus are performed within the inner core.

1.9.3 THE INNER CORE

The inner core contains the minor structural proteins VP1, VP4 and VP6 and the dsRNA genome. The core particle functions as a “molecular engine” by producing full-length capped mRNAs from each of the genome segments. These mRNAs function as templates for viral protein synthesis as well as for negative strand synthesis for the production of progeny dsRNA genome segments (Grimes *et al*., 1998).

For efficient transcription, the dsRNA should be laid down within a pre-existing protein shell. Grimes *et al*. (1998) propose that the minor inner core proteins may attach to VP3 decamers with the result that the complete subcore (VP3, dsRNA genome and minor core proteins) has a transcription complex at each of the 12 five-fold axes. Gouet *et al.* (1999), propose a model for the packing of dsRNA within the inner core of the BTV particle. In their model they suggest that the dsRNA coils around the transcription complex at the five-fold axis (figure 1.1). The transcription complexes are made up of the three minor structural proteins namely VP1 (polymerase), VP4 (capping enzyme) and VP6 (the helicase) (Diprose *et al*., 2001, Mertens and Diprose, 2004).

During transcription, the polymerase utilizes the negative RNA strand as template for the synthesis of a positive daughter strand. The reaction requires NTP and the by-product is pyrophosphate (PPi). The VP4 protein synthesizes a 5’ cap on the daughter strand (Diprose *et al*., 2001). All of the 10 dsRNA segments are transcribed separately and repeatedly (Gouet *et al*., 1999). Each dsRNA segment is thought to be attached to a transcription complex. The dsRNA segments have to move through the polymerase active site during transcription. VP6 (helicase) may be involved in both the unwinding of the parental strands parental-daughter strand complex (fully conservative replication) and export of daughter strands from the core. VP6 uses ATP hydrolysis as energy source producing ADP and phosphate (Pi) as by-products.

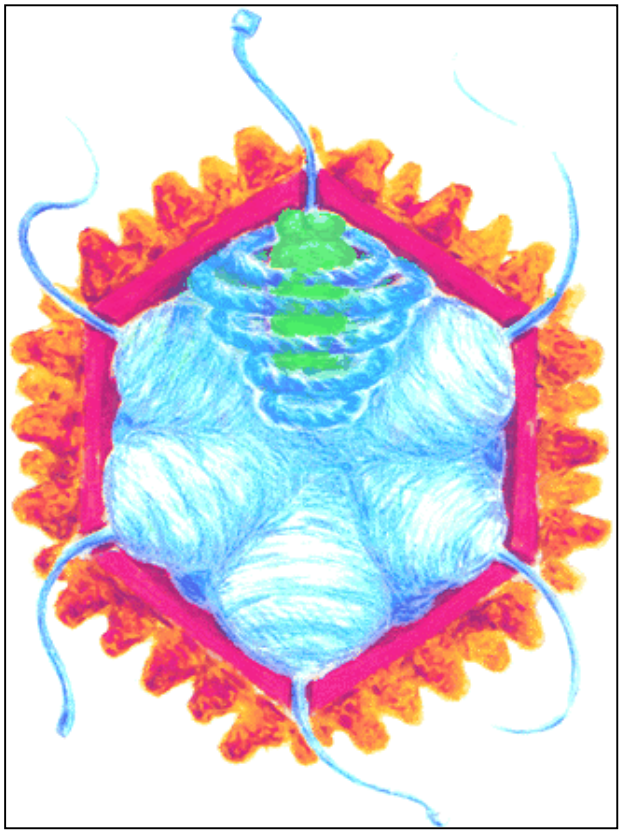


Figure 1.1 Model for the packaging of dsRNA in the BTV core particle.

The RNA is depicted as a blue coil around the transcription complex (in green) showing the position of the RNA with regards to the five-fold vertices (Gouet *et al*., 1999).

mRNAs produced by transcription must be exported from the core for translation in the host cells cytoplasm. Therefore, there must be entry points in the core for NTP and other substrates. Exit points for reaction by-products and mRNA molecules into infected cells are also required. DsRNA must remain inside the core to prevent the host immune response on contact of dsRNA with the cytoplasm of host cell. Diprose *et al*. (2001), have used X-ray crystallography to investigate how the core manages these activities. They have proposed that mRNA is extruded from the core at the pores at the icosahedral five-fold axes. These twelve pores are the largest openings through the VP3 layer. There are positively charged amino acid groups in the five-fold related pores that may guide the RNA by means of electrostatic charge (Grimes *et al*., 1998). The pore between the A and B molecules of VP3 (site N) is used for entry of substrates and release of by-products. This pore carries little overall charge. Further binding sites in the VP7 layer occur between monomers of VP7 between adjacent trimers. Large numbers of nucleotides on the particle surface may act as a substrate sink. This may serve to

increase the local concentration and supply of nucleotides around the core particle thus facilitating polymerase activity. Ion binding sites are located on the core. Two cation sites are found at the base of the VP7 trimers and are occupied by Mg^{2+} ions. A general anionic binding site has been observed between the VP3 and VP7 layers and is occupied by phosphate or sulphate ions. The entry and exit ports are summarized in figure 1.2.

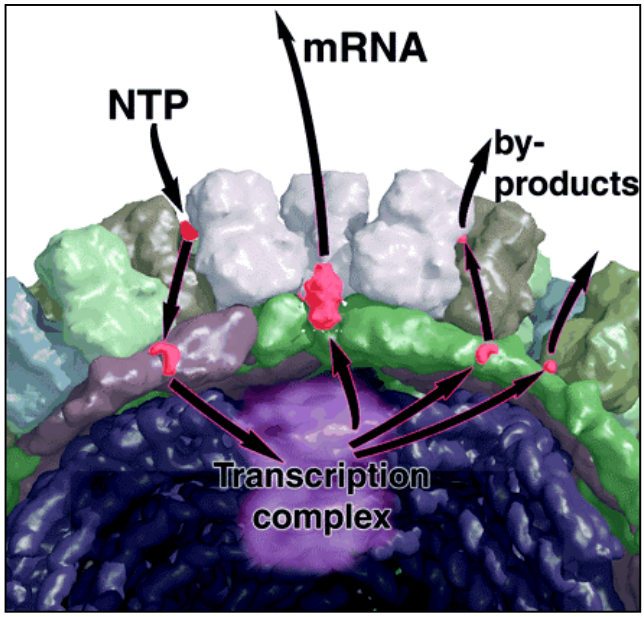


Figure 1.2 Cartoon illustrating the movement of the NTP raw material into the core particle and the site of exit of the mRNA transcript and by-products.

NTPs are sequestered at the inter-trimer site and NTPs are thought to cross the protein barrier between molecules A and B of VP3. The mRNA product leaves via the pore at the five-fold axis of the particle. By-products leave via pores in the VP7 layer at the icosahedral five-fold axes and between molecules A and B of VP3 (Diprose *et al*., 2001).

Most functions in the cell are thought to be carried out by macromolecular complexes or "molecular machines" as opposed to single protein enzymes (Nogales and Grigorieff, 2001). This would seem to be the case in BTV based on the X-ray crystallography derived structural modules. The function of each of the components of the transcription complex of BTV namely, VP1, VP4 and VP6 have been assayed individually.

The putative RNA polymerase VP1 is encoded by segment 1. There is strong amino acid sequence similarity between various RNA and DNA polymerases (Roy, 1992; Huang *et al*., 1995).

VP1 of AHSV has been proposed to include a motif characteristic of RNA-dependent RNA polymerases of positive-strand RNA viruses and some dsRNA viruses (Vreede

and Huismans, 1998). Urakawa *et al*. (1989), demonstrated RNA polymerase activity by means of a poly (U) template -oligo (A) primer polymerase assay for VP1 of BTV. It has further been demonstrated that BTV VP1 has replicase activity, producing dsRNA from plus strand viral template RNA in what appears to be a template independent manner (Boyce *et al*., 2004).

The capping and methylation of the virus mRNA is another enzymatic function performed by VP4 (Roy, 1992). Le Blois *et al*. (1992) demonstrated covalent binding of GTP by VP4 of BTV and proposed VP4 as the candidate guanylyl transferase of the virus. Sequence analysis revealed a potential leucine zipper motif near the C-terminus of VP4 which is conserved among five US BTV viruses (Huang *et al*., 1993). Mutation analysis has revealed that this leucine zipper motif is essential for dimerization of the VP4 molecule which in its native form exists as a dimer in solution. It also appears that dimerization is necessary for assembly of VP4 into BTV cores (Ramadevi *et al*., 1998b). VP4 of BTV has two methyltransferase activities: it caps existing RNA molecules by transferring a methyl group to the guanosine capping residue at the 5' terminal and catalyzes methylation of the ribose of the nucleotide preceding the 5' terminal nucleotide. It is also able to condense GTP to form a capped dinucleotide which confirms its guanylyl transferase activity (Ramadevi *et al*., 1998a). VP4 of BTV has nucleoside triphosphatase (NTPase) activity with a preference for GTP as substrate (Ramadevi and Roy, 1998).

The second smallest RNA segment codes for VP6 which together with VP1 and VP4 make up the mRNA polymerase complex. BTV VP6 is a hydrophilic protein, which is very basic, containing a large number of charged amino acids (Fukusho *et al*., 1989; Roy, 1992). Wade-Evans *et al*. (1992), observed a doublet on expression of VP6 of BTV-1. This doublet has been attributed to two in-phase start codons which supposedly give rise to these two forms of VP6 as opposed to post-translational modification.

VP6 of BTV is a nucleic acid binding protein which binds dsRNA, ssRNA and dsDNA in a mechanism independent of its tertiary structure. In accordance with its nucleic acid binding ability, VP6 is found within the VIBs where virus morphogenesis is believed to occur (Roy *et al*., 1990). Hayama and Li (1994), further characterized BTV VP6 nucleic acid binding activity. By deletion mutation analysis, they localized binding to two domains which correspond to three of six identified antigenic epitopes.

VP6 has long been proposed as the putative viral helicase (Roy, 1992). In BTV, this activity has been demonstrated. It has also been demonstrated to have ATP binding activity and RNA-dependent ATPase activity. Previously unidentified in viral helicases, RNA duplexes with 3' and 5' overhangs as well as blunt-ended dsRNA were unwound by VP6 (Stäuber *et al*., 1997).

Due to its amino acid composition, deletion and substitution mutants of BTV VP6 encompassing the amino and carboxy terminals were generated for investigation of intracellular location and structure-function relationships. This investigation has shown

that residues in the amino terminal are necessary for retention of VP6 in the cell cytoplasm, while residues in the carboxy terminal may allow for nuclear localization. However, the intact protein is located in the cytoplasm (Yi *et al*., 1996).

In a recent study, Kar and Roy (2003), targeted putative helicase domains of BTV VP6. These included sites thought to be involved in ATP binding activity, helicase activity and RNA binding. By site directed mutagenesis, they identified motifs important for ATP binding and hydrolysis as well as RNA binding. According to their results, Kar and Roy (2003) propose that purified BTV VP6 forms multimers which can be stabilized into ring-like particulate structures (hexamers) in the presence of RNA.

1.9.4 THE NONSTRUCTURAL PROTEINS: NS1, NS2, NS3 AND NS3A

There are four virus specific nonstructural proteins found in cells infected with orbiviruses. A characteristic feature of *Orbivirus* infected cells is the polymeric morphological structures assembled from NS1 which is expressed abundantly in infected cells (Huismans and Els, 1979; Roy, 1996). The BTV dsRNA segment 5 codes for the NS1 protein which has a predicted M_r of 64kDa (Lee and Roy, 1987). In BTV, NS1 tubules are found throughout the infection cycle and can be demonstrated at 2 – 4 hr p.i. (Huismans and Els, 1979). These tubules are attached to the intermediate filaments of the cell cytoskeleton (Eaton and Hyatt, 1989) and are associated with virus inclusion bodies (VIB) and virus particles (Eaton *et al.,* 1987). They are thought to be involved in virus transportation in infected cells (Roy, 1996). There is high sequence identity between NS1 within the BTV serogroup (Hwang *et al*., 1993), between serogroups the amino acid identity ranges between 20 and 50% (Moss and Nuttall, 1995). These authors found that five cysteine residues are conserved in AHSV, BRDV, BTV and EHDV. In the case of BTV-10, substitution of two of the conserved cysteines prevents tubule formation (Monastyrskaya *et al.,* 1994). Using a panel of monoclonal antibodies, Monastyrskaya *et al*. (1995) established an NS1 antigenic site near the carboxy terminus which appears to be exposed on the surface of the tubules. In addition, they found that adding 16 amino acids at the carboxy terminus of BTV-10 NS1 does not affect tubule formation. Mikhailov *et al.* (1996), produced various chimeric BTV-10 NS1 genes by inserting foreign sequences at the C-terminus. All of the constructs formed tubules expressing the foreign antigenic sequences on the surface of the tubules and were highly immunogenic, recognizing the native foreign proteins in ELISA. Due to high expression levels and easy purification, NS1 tubules may be a useful system for the presentation of foreign epitopes (Mikhailov *et al.,* 1996). NS1 tubules of AHSV have been characterized (Maree and Huismans, 1997) and are currently under investigation as particulate immunogen delivery systems.

Other virus-specific structures found in cells infected with orbiviruses such as BTV are the virus inclusion bodies (VIBs) (Lecatsas, 1968). A major component of VIBs is nonstructural protein NS2 which is expressed in large amounts in infected cells (Roy, 1996). The assembly of virus particles appears to occur in VIBs. These VIBs contain

structural and nonstructural proteins as well as virus particles at various stages of morphogenesis (Brookes *et al*., 1993). Thomas *et al*. (1990), has reported that baculovirus expressed NS2 of BTV-10 forms inclusion bodies similar to viral inclusion bodies found in BTV infected cells. A comparison of the nucleotide sequences of segment 8 of AHSV, EHDV and BTV showed a similarity of 53 – 60% (van Staden *et al*., 1991). BTV NS2 has been demonstrated to bind ssRNA by means of a nitrocellulose-binding assay where NS2 bound both cellular ssRNA and BTV mRNA (Huismans *et al*., 1987a). Uitenweerde *et al*. (1995) demonstrated that NS2 is a multimer of six or more NS2 molecules. NS2 is a phosphorylated protein which is expressed throughout the virus replication cycle (Huismans *et al*., 1987a). It has been found that NS2 is the only BTV protein that is phosphorylated which is different from equivalent proteins with an affinity for ssRNA in other *Reoviridae* viruses such as reovirus (Huismans and Joklik, 1976) and rotavirus (Boyle and Holmes, 1986). Phosphorylation was found to reduce the efficiency of ssRNA binding of NS2 (Theron *et al*., 1994). Van Staden *et al.* (1991), identified a motif in NS2. This motif plays an important role in the conformation of the protein necessary for ssRNA binding and the formation of VIBs in infected cells (Theron *et al.,* 1996a). It has been proposed that NS2 may have a role in selection and condensation of the virus mRNA (Huismans *et al*., 1987a).

In a study using UV cross-linking assays, NS2 of BTV was found to have the highest affinity for the 3’ regions on viral mRNA. Mutant transcripts lacking 5’ and 3’ highly conserved terminal hexanucleotides bound as effectively as wild-type transcripts. Therefore, these sequences are not necessary for binding (Theron *et al*., 1996b; Theron and Nel, 1997). Three regions with non-specific ssRNA binding activity *in vitro* have been identified in BTV NS2 protein (Fillmore *et al*., 2002). Lymperopoulos *et al*. (2003) have found that BTV NS2 recognizes and binds to a sequence in the coding region of BTV-10 segment 10. They suggest that the secondary structure (hairpin-loop structure with two minor stem-loops in the main-loop area) is important for binding activity. The main stem and part of the main loop are proposed to bind NS2. Horscroft and Roy (2000), have reported NTP binding and phosphohydrolase activity in NS2 of BTV. They have suggested that the NTPase activity may be important in providing energy for selection, transport or packaging of ssRNA.

NS3 and NS3A are the gene products of segment 10 (Mertens *et al.,* 1984; van Dijk and Huismans, 1988; French *et al*., 1989). Peptide mapping of AHSV, Palyam and BTV indicated that they arise from translation of two in-phase start codons (van Staden and Huismans, 1991). The hydrophobic domains, putative transmembrane sequences, possible glycosylation sites and a cluster of proline residues are conserved between BTV and EHDV S10 gene products (Jensen *et al*. 1994). NS3 and NS3A are associated with the plasma membrane of infected cells. They are localized to regions of plasma membrane disruption (Hyatt *et al*., 1991). It was further demonstrated that NS3 / NS3A mediate budding and release of virus-like particles from infected cells (Hyatt *et al*.,

1993). AHSV NS3 has been demonstrated to have a cytotoxic effect on infected cells. It was found to disrupt the cell membrane and immunofluorescence suggested possible membrane association. This membrane association may lead to increased permeability of the cell membrane resulting in the loss of osmotic regulation and subsequent cell death (Van Staden *et al*., 1995).

De Sá *et al*. (1994), investigated the phylogenetic relationships between segment 10 of AHSV serotypes 1, 3, 4, 8 and 9. They found a close relationship between serotypes 4 and 9; 1 and 8 and serotype 3 was found to be closer to the 4 and 9 group. Higher variation across and within serotypes of NS3 of AHSV was found than in EEV and BTV NS3. These findings indicate that NS3 is the second most variable AHSV protein after VP2 (Van Niekerk *et al*., 2001b; Van Niekerk *et al*., 2003). Van Staden and Huismans (1991) reported two highly conserved regions in the amino acid sequence of NS3 of serotypes 3 and 9. These were found to be conserved in all five serotypes (De Sá *et al*., 1994). Comparisons of the hydrophobic profiles of AHSV and BTV NS3 revealed two conserved hydrophobic domains (De Sá *et al*., 1994). Van Staden *et al*. (1995) identified these two hydrophobic domains as regions capable of forming transmembraneous helices in BTV, AHSV, Palyam, BRD and EHDV. Using this data, they proposed a model for the membrane associated topology of NS3 with the transmembrane regions spanning the membrane and the N and C termini on the cytoplasmic side of the membrane. Some support for this model was provided by an investigation of membrane organization of bluetongue virus NS3 (Bansal *et al*., 1998). Their data indicated that both the hydrophobic domains span the cell membrane with the likelihood that the N and C termini are cytoplasmic.

Mutations in either of the hydrophobic domains destroys membrane anchoring of NS3 of AHSV (van Niekerk *et al*., 2001a). As a result of this, NS3 is no longer localized to the cell surface and the cytotoxic effect is eliminated. Van Niekerk *et al*. (2001a) have demonstrated that cytotoxicity of NS3 is dependent on membrane association determined by both the hydrophobic domains.

BTV NS3 / NS3A exist as N-linked glycoproteins (Wu *et al.,* 1992). Bansal *et al*. (1998) have demonstrated that only one of the two glycosylation sites in bluetongue virus NS3 is utilized. Using mutants in which the active glycosylation site is deleted, no NS3 could be detected in association with the cell membrane. They suggest that this may be as a result of failure of transport due to improper folding or proteolysis resulting from the lack of protection by the carbohydrate.

In a study using an avirulent and a virulent strain of African horse sickness virus, three phenotypes were observed in a mouse model namely, fully virulent, fully avirulent and a new intermediate virulence phenotype. The intermediate phenotype arose from reassortment of segment 10 between the avirulent and the virulent strains (O'Hara *et al.*, 1998).

1.10 VIRAL ENZYMATIC FUNCTIONS

Virus replication is performed using the host cell machinery. As a result of this, viruses contain a few enzymes at most. The genomes of double-stranded nucleic acid viruses function as templates for replication and transcription (Fields, 1998).

The synthesis of mRNA in BTV is performed by the RNA polymerase associated with the core particle (Huismans and van Dijk, 1990). Replication and transcription are initiated after unwinding of the double-stranded nucleic acid occurs. After virus entry into permissive cells, the outer capsid layer is removed and transcriptase activity occurs inside the viral core (Huismans and van Dijk, 1990; Stäuber *et al*., 1997). Capped and methylated mRNA extrudes from the viral core to be translated into viral proteins in the cytoplasm of infected cells (Huismans and van Dijk, 1990; Patton *et al*., 1990; Roy, 1992; Stäuber *et al*., 1997; Diprose *et al*., 2001).

1.10.1 VIRAL HELICASES

DNA helicases, first reported in 1976 (Abdel-Monem *et al*., 1976) and RNA helicases, first reported by Lain *et al.* in 1991, are found in both prokaryotes and eukaryotes. Helicases enzymatically unwind duplex nucleic acid structures of cellular and viral genomes by translocation along one strand (Geider and Hoffmann-Berling, 1981; Gibson and Thompson, 1994). They generate single-stranded intermediates which are required for replication, recombination and repair (Matson and Kaiser-Rogers, 1990; Runyon and Lohman, 1993; Lohman and Bjornson, 1996). Helicase activity is linked to NTP hydrolysis which presumably provides energy for the reaction. The reaction also requires the presence of a divalent cation, usually Mg^{2+} (Gomez de Cedron *et al*., 1999; Bideshi and Federici, 2000). All helicases described to date have NTPase activity (Matson and Kaiser-Rogers, 1990; Lohman and Bjornson, 1996; Kadaré and Haenni, 1997).

1.10.1.1 Structural features of helicases

Mechanisms for helicase action propose two features: ATP-driven conformational rearrangement that generates energy and a minimum of two nucleic acid binding sites between which energy can be employed to execute unwinding (Gibson and Thompson, 1994; Kadaré and Haenni, 1997). The two sites would allow intermediates to bind either two single-stranded nucleic acid regions or one single-stranded and one duplex region at an unwinding junction. Evidence for such intermediate complexes exists for the Rep protein of *E. coli* (Lohman, 1992; Lohman, 1993).

The most direct mechanism for a helicase to obtain multiple nucleic acid binding sites is oligomerization (Lohman and Bjornson, 1996). Enzymes that are monomeric in solution may assume active oligomeric forms upon binding of a nucleic acid or a nucleotide cofactor. An example is the Rep helicase of *E. coli* which dimerizes only on DNA binding (Lohman, 1992; Lohman, 1993; Kadaré and Haenni, 1997).

DNA helicases can be divided roughly into two groups namely: hexameric helicases and non-hexameric helicases. Recent structural and biochemical data has shown that non-hexameric helicase bacteriophage T4 helicase Dda is active in a monomeric form (Marians, 2000).

Comparisons of amino acid sequences of proteins involved in nucleic acid metabolism have yielded several different motifs. These motifs include ATPase activity, helicase activity, NTP and nucleic acid binding sites (Matson and Kaiser-Rogers, 1990; Kadaré and Haenni, 1997).

1.10.1.2 Helicase families

Based on sequence comparisons, helicase proteins may be divided into five major groups. These superfamilies (SF) include cellular and viral DNA and RNA helicases (Lohman and Bjornson, 1996; Kadaré and Haenni, 1997, Soultanas and Wigley, 2001).

The superfamilies are classified as follows: in SF1 are the alphavirus-like nsP2 proteins, this includes nsP2 of Semliki forest virus (Gomez de Cedron *et al*., 1999). The SF1 proteins are associated with a zinc finger structure (Seybert *et al*., 2000; van Dinten *et al*., 2000). The helicase of vaccinia virus and proteins encoded by the potyvirus-flavivirus-pestivirus NS3-like proteins such as the flavivirus, West Nile virus NS3 (Borowski *et al*., 2001) are found in SF2. SF2 includes the DexH/DEAD protein family (Koonin, 1991, Gibson and Thompson, 1994). SF1 and SF2 are characterized by seven conserved motifs (Caruthers and McKay, 2002). SF3 includes picornavirus-like 2C-like proteins. It also includes the parvovirus NS1. SF3 proteins have three conserved motifs including the “A” and “B” sites and a third site designated “C” (Kadaré and Haenni, 1997). Helicases that are related to the DnaB protein of *E. coli* fall into the fourth group. These helicases usually form hexameric ring structures and have five conserved motifs. The fifth family was recognized as having sequence similarity to the β subunit of proton-translocating ATPases, represented by the transcription termination factor Rho (Caruthers and McKay, 2002, Singleton and Wigley, 2002). Proteins are usually classified into one of the above families on the basis of characteristic helicase motifs. Some proteins have been biochemically investigated. It is becoming evident that only a small percentage of these proteins actually possess helicase activity. This may be because the helicases are only functional when part of a protein complex. Another explanation is that these helicase motifs are characteristic of NTP dependent nucleic acid translocases (Soultanas and Wigley, 2001; Singleton an Wigley, 2002). The translocase molecular motor may not be able to unwind duplex nucleic acid. Helicase activity may be conferred by other extra protein domains, excluding the helicase motifs (Caruthers and McKay, 2002).

Certain helicases, for example RuvB, belong to the AAA^+ family rather than the previously described helicase super families (Singleton and Wigley, 2002). The AAA^+ family is thought to represent a broad class of mechanoenzymes which in many cases form hexameric rings that change their conformation during the ATPase cycle. Amongst

a large array of functions, some AAA^+ proteins are molecular motors with ATP binding sites (Vale, 2000).

In 1990, Dorer and others reported the discovery of a new member of the RNA helicase family. This helicase of *Drosophila* differs from the SF helicases in that it has glycine repeats. Glycine rich proteins (GRPs) have been described in numerous organisms including RNA binding proteins (Sachetto-Martins *et al*., 2000). Most helicases that have glycine repeats or are glycine rich have other characteristic motifs such as DEAD/DexH, RNP-1 and RNP-2 or dsRBDs, (Roussell and Bennet, 1993; Sato, 1994; Zhang and Grosse, 1997). Glycine rich sequences have been reported to play a role as anchors for ATP (Bossemeyer, 1994).

1.10.1.3 Function of the conserved motifs

The conserved motifs in helicases may divided according to primary function. Some are primarily involved in binding MgATP/MgADP, others bind oligonucleotides and there are those that play a role in coupling the ATPase cycle to the intramolecular conformational changes involved in the unwinding and strand displacement activities (Caruthers and McKay, 2002).

The most obvious common factor of the superfamilies is the NTP binding motif. This motif consists of the "A" and "B" sites of the "Walker box". "A" may be [$GxxxxGK^S/_T$, where x is any amino acid] or the abridged [$GxGK^S/_T$] (Gorbalenya *et al*., 1989). It forms the P-loop within the NTP binding site. "B" consists of an aspartate (D) which interacts with the NTP by means of Mg^{2+} (Lohman and Bjornson, 1996; Kadaré and Haenni, 1997). The NTP binding motif alone is not sufficient to propose a helicase signature as there are a variety of proteins that utilize NTP but are not necessarily helicases. The energy generated by NTP hydrolysis may be used for processes such as RNA encapsidation, intercellular movement of the virus or vesicular trafficking of replication complexes (Kadaré and Haenni, 1997). Another important aspect of helicase activity is the ability to bind nucleic acids.

Numerous nucleic acid binding motifs have been identified for both DNA and RNA binding proteins. Focusing on RNA binding motifs, possibly the most common and best characterized RNA binding motif is the RNP (or otherwise known as the RNA recognition motif - RRM) which consists of the RNP-1 ((K/R)G(F/Y)(G/A)FVX(F/Y)) and RNP-2 ((L/I)(F/Y)(V/I)(G/K)(G/N)L)) sites (Burd and Dreyfuss, 1994; Sachetto-Martins *et al*., 2000). Another common motif is the arginine-rich motif (ARM). It is characterized by 10 – 20 amino acids, rich in arginine (Mattaj, 1993). The RGG box has 20 – 25 amino acids with RGG repeats which are closely spaced with other (often aromatic) amino acids. Usually the RGG box is found in combination with other types of RNA binding domains. Binding is generally sequence non-specific (Burd and Dreyfuss, 1994). The KH motif is a complex motif identified in the hnRNP K protein. The KH motif proteins of known function are associated with RNA and bind RNA in vitro (Mattaj, 1993; Burd and Dreyfuss, 1994). The double-stranded RNA binding motif (dsRBM) consists of a 70

amino acid region that binds double-stranded RNA characterized by an α-β-β-β-α fold. There are nine families of proteins with the dsRBM. The proteins are classified according to their number and distribution of dsRBMs. Other additional domains characterize these protein families (Fierro-Monti and Mathews, 2000). These proteins are involved in diverse cellular functions. The Zinc finger-knuckle ($CX_{2-5}CX_{4-12}C/HX_{2-4}C/H$) is found in a small number of RNA-binding proteins which include RNA polymerases and retroviral nucleocapsid proteins (Burd and Dreyfuss, 1994). The cold shock domain (CSD) has been found in RNA binding proteins such as CspA and CspB. The RNA binding domain has conserved aromatic and basic residues and binds dsDNA, ssDNA and RNA (Graumann and Marahiel, 1996). Helicase motif VI (RXGRXXR), characteristic of SF2 helicases, has an important role in RNA binding (Kadaré and Haenni, 1997; Kim *et al*., 1997; Kar and Roy, 2003). This motif is characterised by a high number of positively charged amino acid residues. Finally, GRPs (Glycine-rich proteins) are a family of proteins found in many different organisms, they are proposed to be involved in protein-protein interactions. The glycine-rich regions are thought to result in a highly flexible secondary structure. They are often found in association with RNP-1 and RNP-2. The consensus motif may be GGGX; GGXXXGG or GXGX (Sachetto-Martins et al., 2000). Bossemeyer (1994) reported two consensus sequences found in glycine rich nucleotide binding proteins. $GxGxGL^{S}/_{T}$ is usually found in proteins with a preference for binding mononucleotides. GxGxxG on the other hand may be found in proteins that bind dinucleotides preferentially.

1.10.1.4 Helicase activity

NTPase activity of helicases is generally stimulated by the presence of single-stranded nucleic acids, preferentially by the type of nucleic acid to which the helicase is functionally related (i.e. DNA or RNA) (Lohman, 1993; Kadaré and Haenni, 1997). Characterized RNA helicases hydrolyze ATP in the presence of different RNAs including synthetic homopolymers and also single-stranded DNA. They all bind single-stranded RNA and DNA. It was thought that most RNA helicases cannot unwind DNA, however, notable exceptions include vaccinia virus NPHII (Bayliss and Smith, 1996) and SV40 T antigen (Stahl *et al*., 1986; Scheffner *et al*., 1989) both of which may act as DNA or RNA helicases depending on the context, eIF-4A (Matson and Kaiser-Rogers, 1990; Kadaré and Haenni, 1997) Hepatitis C NS3 (Gwack *et al*., 1997) and λ1 protein of Reovirus (Bisaillon *et al*., 1997). Some DNA helicases such as the Rho protein of *E. coli* are capable of unwinding RNA/DNA hybrids in an ATP dependent manner. This type of reaction could be significant in pre-mRNA splicing, translation and other cellular processes (Matson and Kaiser-Rogers, 1990). There is evidence indicating that some helicases may be sensitive to the structure of the duplex substrate. For example, in unwinding studies using hepatitis C virus (HCV) NS3 it was found that heteroduplexes of peptide nucleic acid (PNA) and DNA or RNA adopt a structure that is unfavourable for unwinding by NS3 of HCV. While morpholino-DNA and phosphorothioate-DNA and DNA-DNA substrates were utilized efficiently (Tackett *et al*., 2001).

During helicase activity, the helicase enzyme remains bound to one strand using the energy generated by ATP hydrolysis to disrupt hydrogen bonding of a duplex structure. It is thought that a protein conformation leading to optimized binding and hydrolysis of the NTP may be induced by nucleic acid binding. High salt concentrations do not stimulate NTPase activity suggesting that in this condition the enzyme may not remain bound to the nucleic acid (Kadaré and Haenni, 1997) or, as in the case of the T-antigen, helicase of SV40, the enzyme assumes a conformation not suitable for helicase activity (Scheffner *et al.*, 1989; Kadaré and Haenni, 1997). Most DNA helicases function either 5' to 3' or 3' to 5' (Matson and Kaiser-Rogers, 1990). The polarity of an unwinding reaction is defined by convention with respect to the strand of nucleic acid to which the helicase is bound (Matson, 1991). *E. coli* RecBCD which is a recombination helicase, unwinds blunt ended DNA duplexes and is inhibited by flanking ssDNA regions that exceed 25 nucleotides. The RecB subunit interacts with the 3' end of the ssDNA strand and the RecC subunit interacts with the 5' end of the ssDNA strand. This provides direct evidence for helicase interaction with both strands. There is no direct evidence supporting strict unidirectional translocation of a helicase even though it may preferentially unwind DNA with a 3' or 5' ssDNA flanking region. A functional initiation complex may only be formed when an appropriate single-stranded flanking region is available (Lohman, 1993). RNA helicases described up to 1997 such as the helicase of tamarillo mosaic potyvirus and vaccinia virus helicase unwind unidirectionally 3' to 5' (Shuman, 1992; Eagles *et al*., 1994; Kadaré and Haenni, 1997). However, as mentioned above, RNA helicases such as VP6 of BTV possess bi-directional helicase activity as well as the ability to unwind blunt ended substrates (Stäuber *et al*., 1997). Binding of a helicase to a region of unpaired nucleic acid was thought to be a prerequisite for the initiation of the unwinding activity (Geider and Hoffman-Berling, 1981), this, however, is clearly not the case with BTV VP6 (Stäuber *et al*., 1997).

1.10.1.5 Models of helicase activity

Several mechanisms for DNA unwinding and translocation have been proposed. These may be active or passive depending on whether the helicase plays a role in the unwinding or whether it functions only to stabilize the ssDNA. According to the passive mechanism, the helicase sequesters single-stranded nucleic acid that arises as a result of thermal fluctuations at single-stranded/double-stranded nucleic acid junctions. Based on kinetic measurements of duplex DNA unwinding, NS3 of hepatitis C virus (HCV) has been proposed to function using a passive mechanism (Porter, 1998). However, other researchers have concluded that NS3 of HCV interacts weakly with the displaced strand. Therefore, conclusive evidence for a passive mechanism is not available (Paolini *et al*., 2000; Tackett, *et al*., 2001).

Two types of active helicase mechanism have been proposed. The first type is the "torsinol" model. In this model, the helicase binds to both ssDNA strands at the ss/dsDNA junction with no interaction with the duplex. Unwinding occurs as a result of the distortion of the adjacent duplex region through NTP induced conformational

changes. The other type of active unwinding mechanism includes both the “inchworm” and “rolling” models. In the inchworm model, there are two different binding sites: one site binds with polarity to ssDNA while the other site (the leading site) binds both single-stranded and dsDNA. Translocation occurs by means of conformational changes coupled to the binding and hydrolysis of one or more NTP molecules. The enzyme travels along the dsDNA and separates the duplex in the fashion of a snowplough. This model has been proposed for PcrA, a monomeric helicase, based on crystal structures of PcrA bound to a DNA substrate (Bird *et al*., 1998; Velankar *et al*., 1999). The “inchworm” model is illustrated in figure 1.3.

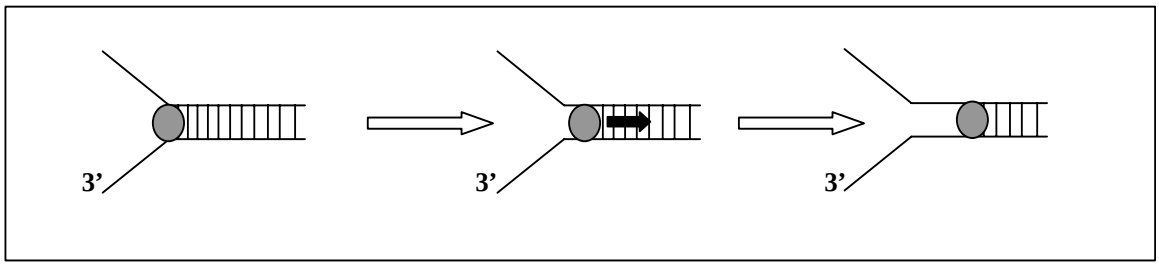


Figure 1.3 The inchworm model of helicase activity.

The helicase molecule (represented by the grey circle) moves along the DNA in discrete steps “melting” the DNA strands as it goes. The helicase in this model does not have to be oligomeric (redrawn from Bird et al., 1998).

The “rolling” model is based on the Rep molecule of *E. coli*. This model is based on observed allosteric effects of nucleotide cofactors on the single-stranded and dsDNA binding properties of the Rep dimer. It could be applied to any homo-oligomeric helicase. One subunit of the Rep dimer is always bound to 3’ ssDNA at the fork while the other may either bind to the same single strand or to the adjacent duplex region ahead of the fork. Each subunit alternates between binding of single-stranded and dsDNA controlled by the allosteric effects of ATP and ADP binding. In this model the Rep dimer translocates by rolling along the DNA with translocation coupled to ATP binding and DNA unwinding coupled to ATP hydrolysis (Lohman, 1993; Lohman and Bjornson, 1996). The “rolling” model is illustrated in figure 1.4.

Of the various models proposed, work on the SF1 and SF2 enzymes (usually monomeric) have suggested that the inchworm model may be the most likely helicase mechanism (Soultanas and Wigley, 2000, Singleton and Wigley, 2002). There is some debate on whether the mechanism is active or passive with regards to ATP hydrolysis. Is the energy derived from hydrolysis utilized for translocation and duplex separation or translocation alone? There is considerable thermal fraying of duplex DNA at room temperature. However, the RecBCD helicase translocates at a rate excessive of 1kb/s which is thought to be faster than passive duplex fraying (Singleton and Wigley, 2002).

Recently, equilibrium binding studies have demonstrated that the HCV helicase single-stranded binding activity is increased by the presence of ADP and the absence of ATP (Levin *et al*., 2003). When the HCV helicase bound ATP, a hundred fold reduction in its affinity for ssDNA was observed. This has not been observed for duplex DNA. These results would appear to suggest that the nucleic acid binding site of the HCV helicase is allosterically modified by the ATPase reaction. The binding energy that is used to change the conformation (brings the HCV helicase out of a tightly bound state) may be used to facilitate translocation. The ATP hydrolysis reaction and product release are thought to promote tight rebinding of the nucleic acid and the helicase. Levin *et al.* (2003), propose a Brownian motor model for the unidirectional translocation of HCV helicase along the length of the nucleic acid strand.

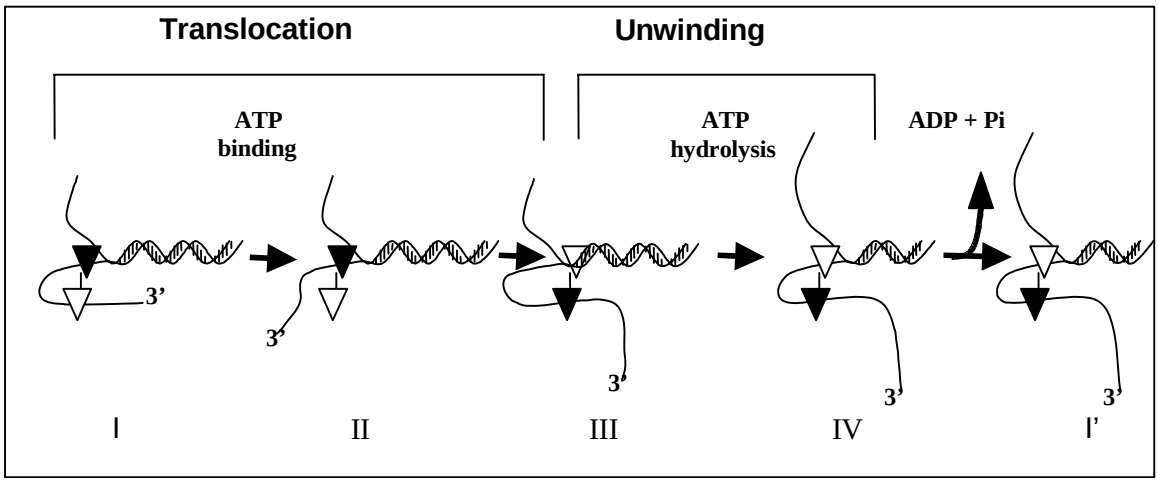


Figure 1.4 "Rolling" mechanism for Rep-catalyzed DNA unwinding.

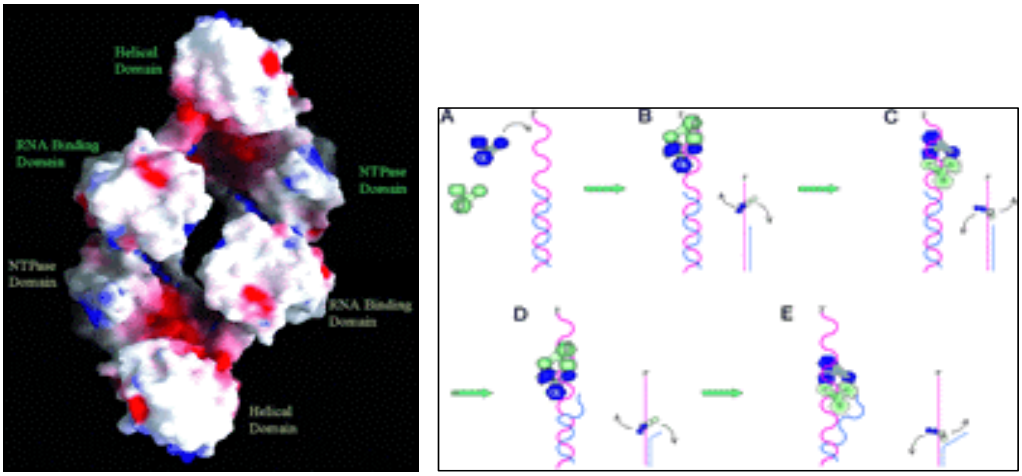
A dimeric helicase in which the subunits possess C2 symmetry, unwinds by direct interaction with both ssDNA and dsDNA. I is an intermediate where both subunits are bound to the 3' single strand. When ATP is bound, the affinity of one subunit for ssDNA decreases and the intermediate II is formed. As its affinity for ssDNA decreases, its affinity for dsDNA increases resulting in the formation of intermediate III. In steps III – IV, ATP hydrolysis brings about a protein conformation change that results in destabilization of basepairing within a region of duplex DNA bound to the Rep protomer. As a result, the 5' single strand is displaced while the Rep protomer remains bound to the 3' strand. The cycle is completed with the release of ADP and inorganic phosphate by the formation of the I' complex. This is functionally equivalent to intermediate I because Rep is a homodimer (Lohman, 1992; Lohman, 1993; Bird *et al*., 1998).

Progress towards a more complete picture of the structure and mechanism of helicases has recently come from high resolution crystallography. The crystal structures for a number of helicases have been determined. The structural similarities between

hexameric and non-hexameric helicases suggest the possibility of a single mechanism (Bird *et al.*, 1998; Soultanas and Wigley, 2000).

Cho *et al.* (1998), determined the crystal structure of the RNA helicase domain of HCV type 1b at 2.3 Å resolution. The oligomeric state of a functional HCV helicase is unknown. These authors investigated the packing interfaces and propose a “symmetry-related monomer-monomer” interaction possibly reflecting a functional form of the RNA helicase. Interfaces arise from contacts between the NTPase and RNA binding domains of the two molecules. The RNA binding motif of each molecule forms a helical shaped channel in the middle of the dimer (figure 1.5a).

Cho *et al*. (1998), have proposed a ‘descending molecular see-saw’ model for HCV NS3. This model is consistent with properties previously observed for the protein. According to their model, duplex RNA is unwound coupled with NTP hydrolysis. As ATP hydrolysis results in the dissociation of RNA from the enzyme, it is hypothesized that NTP hydrolysis causes a hinge bending motion. This transforms the enzyme from its activated conformation to its resting conformation coupled with the release of the bound ssRNA from the interdomain cleft of the resting molecule of the proposed dimer. The dimer thus rotates relative to the bound ssRNA. The rotation axis must be towards the 5’ end of the bound RNA in order for the dimer to be able to translocate on the ssRNA. In this model, the RNA binding motifs are thought to play a very important role in the helicase mechanism by serving as the pivot region for the rotation. The dimer rotates so that the resting molecule goes into the activated conformation. Repeated cycles of rotation and translocation are described as a “descending molecular see-saw” (figure 1.5b).



(a) (b)

Figure 1.5 Crystal packing interaction of the crystals of HCV RNA helicase

a) ssRNA is thought to pass through the channel created by the two molecules. There is a 2-fold crystallographic symmetry axis approximately perpendicular to the figure. The interdomain cleft shown in red is thought to be mostly negative in charge. The dimer shown above is proposed to be similar in conformation to the putative active dimer. (b) Schematic diagram of the 'descending molecular see-saw' model for the translocation of HCV RNA helicase along the ssRNA. In (A) the α helicase molecule binds ssRNA. In (B), another helicase molecule, β, binds and forms a functional dimer. A small conformational change (induced by the ssRNA bound to the interdomain cleft of α) is thought to increase the NTP hydrolysis activity. In (C), as a result of NTP hydrolysis the ssRNA detaches and the dimer rotates at the RNA binding motif of α. Thus the dimer translocates in a 5' direction on the ssRNA. β binds the ssRNA at its interdomain cleft. In (D), the point where the ssRNA becomes dsRNA is reached by repeated cycles of translocation. In (E), interactions between the interdomain cleft and the ssRNA provide energy for the disruption of basepairs. The strand moving through the channel, generated by the dimer interface, is separated from the strand that hangs out of the dimer (Cho *et al*., 1998).

The first high resolution X-ray data on a hexameric helicase (2.4 Å resolution) was provided for RepA of plasmid RSF1010 (Niedenzu *et al*., 2001). The RepA helicase is a DNA helicase that forms a hexamer in its functional form. It has an annular shape with a central hole and 6-fold rotational symmetry (C6). The hexamer has a positive surface charge with negatively charged areas restricted to the bottom side of the central hole. The size of the central hole only allows ssDNA to thread through the hexamer (figure 1.6).

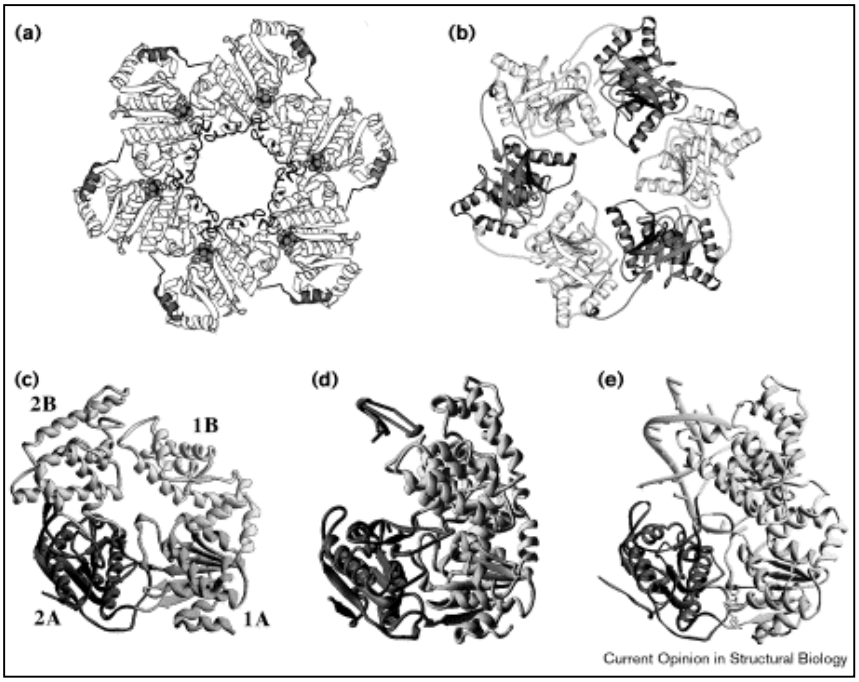


Figure 1.6 Crystal structures of two hexameric helicases

a) T7 helicase domain from the helicase-primase protein and b) the RepA hexameric ring. Both are viewed parallel to the 61 crystallographic axis clearly illustrating the six molecules forming the crystallographic unit. c) – e) Represent the three forms of the monomeric helicase PcrA. The four different subdomains identified by Velankar et al., (1999) are indicated on the apo form (c). d) Product form and e) is the substrate complex. (Soultanas and Wigley, 2000).

Two different structures of PcrA DNA helicase complexed with a DNA duplex with a ssDNA tail have been used to propose a model for a general helicase mechanism (figure 1.7) (Velankar *et al*., 1999). This model is loosely based on the 'inchworm' model and may be divided into two processes namely, translocation and duplex destabilization. These two processes together are responsible for helicase activity. When the helicase binds to ssDNA, translocation begins. Domain swivelling is initiated which allows binding of a DNA duplex. At this stage, ssDNA is bound to domains 1A and 2A (figure 1.6c). The cleft between the two domains closes when ATP is bound. For this to occur, one of the domains (2A) releases its hold on the ssDNA. The bound ATP

is hydrolized and the cleft opens. The helicase moves along the DNA across domain 2A while domain 1A maintains its hold on the DNA. This continues until the helicase reaches a duplex. The protein has a greater affinity for dsDNA and accordingly binds to the duplex region next to the fork. The duplex DNA is dragged onto the surface (domains 1B and 2B figure 1.6c) and there is pressure on the basepairing at the fork. The structure of the dsDNA becomes distorted and several basepairs near the fork are destabilized. Therefore, binding energy generates more ssDNA along which the helicase can translocate. This model (otherwize referred to as the “Mexican wave model” figure 1.7b) has several implications for the understanding of helicase activity. According to this model, helicase activity is active rather than passive. Components required for DNA translocation are found in domains 1A and 2A which are widely conserved in RNA and DNA helicases. Helix destabilization and/or substrate specificity is controlled by domains 1B and 2B which are considerably more variable. In this model, domain swivelling creates a site for binding dsDNA once the protein is bound to ssDNA. Thus the helicase cannot interact with dsDNA facilitating strand separation within a sealed duplex because it must be activated by the presence of ssDNA. This model may be a general model for helicase activity (Velankar *et al*., 1999; Soultanas and Wigley, 2000).

1.10.1.6 Prevalence and role of helicases in viruses

There appears to be a correlation between the genome size of a virus and the presence of a putative helicase. For example, birnaviruses which have small genomes have no known helicase motifs (Koonin, 1991). All dsDNA viruses, for which complete sequence data is available, contain helicase motifs. RNA viruses with large genomes such as reoviruses (Harrison *et al*., 1999) have helicase motifs. Genome sequences of single-stranded viruses have yielded putative helicases. The genomes of single-stranded viruses may be partially duplex in form. During replication of positive stranded RNA viruses, templates must be available for the next round of replication. It has, therefore, been proposed that single-stranded viruses may require helicases to disrupt intramolecular base pairing in template RNA and to prevent extensive base pairing between template and complementary strands (Kadaré and Haenni, 1997).

Three general hypothesis regarding the functions of helicase enzymes in viral life cycles have been proposed. Firstly, helicases may have a proof reading energy dependent mechanism responsible for the fidelity of replication. This may explain why viruses with large genomes (larger than 5kb) encode putative helicases, as they are needed for efficient replication (Koonin, 1991).

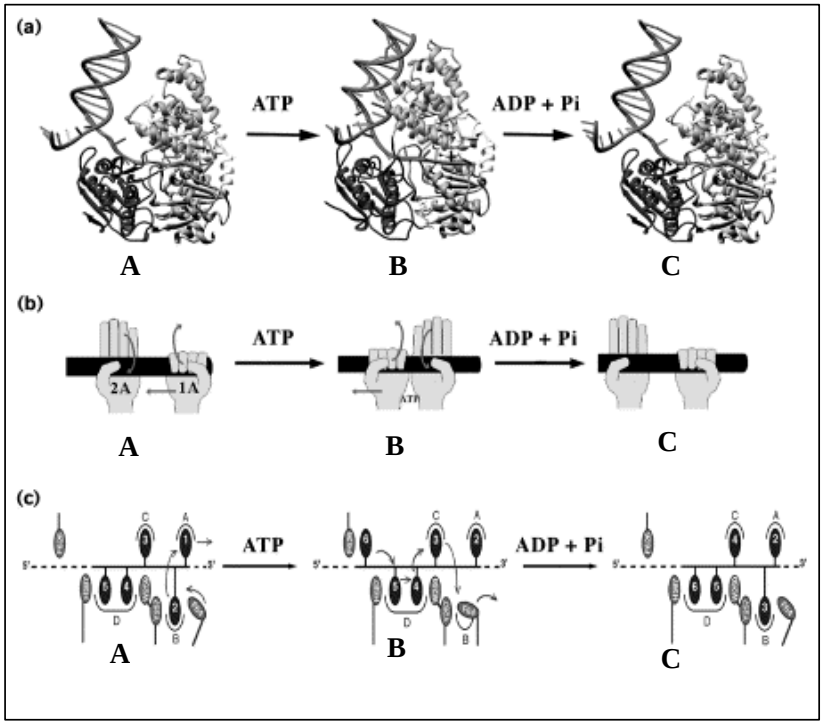


Figure 1.7 Model for helicase activity based on changes in the conformation of both the protein and nucleic acid substrate

(a) The intermediates are based on the three forms of PcrA (Velankar et al., 1999)(figure 1.6 c – e). In step A, the helicase binds to the ssDNA. In step B, following ATP binding, a conformational change in the protein allows it to bind dsDNA at domains 1B and 2B. This is coupled with the disruption of several basepairs at the fork. In step C, after ATP hydrolysis, the protein conformation reverts to that in (A) and the helicase moves along the ssDNA by one base and lets go of the dsDNA. (b) Cartoon showing the alternating ssDNA affinity of domains 1A and 2A. A loose grasp of the ssDNA is illustrated by an open hand while a closed hand represents a tight grip. (A – C reflect the same events in (a); (b) and (c)). (c) Cartoon illustrating the ssDNA binding region at each stage showing the conformational changes as the bases move between binding pockets when the helicase translocates along the ssDNA (Velankar *et al*., 1999; Soultanas and Wigley, 2000).

According to the second hypothesis, helicase activity may be required for strand separation of double-stranded structures during transcription and prevention of R-loop formation behind the elongating RNA polymerase. This activity may be necessary for the initiation of (early) transcription and has been proposed for vaccinia virus helicase (Gross and Shuman, 1996). The third hypothetical role for virus encoded helicases is during initiation of translation. This hypothesis is based on eIF-4A. Together with eIF-4B, eIF-4A is thought to disrupt secondary mRNA structures upstream of the initiation codon thus facilitating 40S ribosome attachment (Matson and Kaiser-Rogers, 1990; Rozen *et al*., 1990). It is sometimes difficult to determine unambiguously which process

a specific helicase is involved in as many organisms encode multiple helicases such as *E. coli* which has at least 10 helicases (Matson, 1991; Lohman, 1993).

1.10.1.7 Past and future aspects of viral helicases

The signatures of the helicase super families are found in both prokaryotic and eukaryotic helicases. This suggests that their common ancestor existed very early in the evolution process and the main branching point may possibly have occurred prior to the eubacterial/eukaryotic divergence. For this reason, it has been proposed that the helicases of the SFs form a fairly compact domain within the large number of proteins with NTP-binding motifs (Kadaré and Haenni, 1997; Anantharaman *et al*., 2002).

Comparison of the three dimensional structures of ABC (ATP-binding cassette) transporters and helicases have shown that there are similarities in the structure of their nucleotide binding sites, their ATP binding domains and the positioning of important residues. It is possible that these two protein families share a common mechanism for harnessing the energy produced by ATP hydrolysis. This may indicate a common ancestor and the two families may be considered to be closely related (Geourjon *et al*., 2001).

Regions of DNA involved in helicase binding can theoretically be identified by Dnase I and chemical protection methods (i.e. footprinting). However, unless there is high specificity for helicase binding, it may be difficult to distinguish between productive and non-productive complexes. As a result, determining functional contacts is difficult. A case in point is SV40 large T antigen. Two different groups applied DNA footprinting techniques and obtained different results (Lohman and Bjornson, 1996). In the case of viral RNA helicases, no specific RNA sequence requirements have been reported. However, it is presumed that such specificity does exist *in vivo* and may arise from a combination of sequence, secondary and tertiary structure of the RNA substrate (Mattaj, 1993; Kadaré and Haenni, 1997). Helicases and their substrates may offer targets for drug development specifically with a view to interfering with viral infection cycles as these proteins appear to fulfil important roles for the virus (Kadaré and Haenni, 1997). In particular, NS3 of hepatitis C virus which has both protease and helicase activities is considered an important target for anti-HCV drug therapy (Tackett *et al*., 2001). Borowski *et al*. (2002), have suggested possible mechanisms for using the NS3 of the hepatitis C virus for antiviral therapy. NTPase activity may be inhibited by the introduction of a competitive NTPase inhibitor, for example one which blocks the ATP binding site and inhibits the ATPase activity of the enzyme. Alternatively, allosteric interference may be used to inhibit unwinding by sterically blocking translocation. NTPase and helicase activities may be uncoupled by disrupting the energy transfer within the helicase. Competitive inhibition of RNA binding, for example, RNA aptamers with many secondary structures interact with the HCV helicase NS3 protein and stop helicase activity. Intercalating agents stabilize both DNA and RNA and, therefore, the energy required for unwinding is higher.

Helicase proteins differ in size and may have additional N- and C-terminal sequences that determine the specific action of each protein. These sequences may include membrane targeting signals, nuclear localization signals or regions for interaction with modulating accessory proteins (Kadaré and Haenni, 1997). This may explain the diversity of functions that are achieved by variations on the common structural theme. In some viruses, different enzymatic functions are assigned to different proteins as is the case with BTV, AHSV and other orbiviruses. In other viruses, helicases may function in complexes with other proteins (Lohman, 1993). For example, in brome mosaic virus (Ahola *et al*., 2000), one protein is responsible for RNA capping and helicase activity, while a different protein is related to polymerases. A similar situation exists for λ1 protein of Reovirus (Bisaillon and Lemay, 1997). NS3 of hepatitis C virus has both protease and helicase functions and is thought to be involved in viral replication (Jin and Peterson, 1995; Banerjee and Dasgupta, 2001). The function of viral enzymes is to ensure that viral proteins are expressed and new virions are produced. This involves the processes of transcription and replication. Together with helicases, important viral enzymes include polymerases and mRNA capping enzymes.

1.10.2 VIRAL TRANSCRIPTASE ACTIVITIES

In the case of Reovirus, the λ1 protein has NTPase and helicase activity as well as 5' RNA triphosphatase activity. Besides its role in transcription of the viral genome, λ1 protein is thought to participate in the capping of the 5' ends of new viral mRNAs. Capping involves the removal of the 5' end phosphate by means of the polynucleotide phosphohydrolase function of the RNA triphosphatase. GMP derived from GTP is donated by guanylyltransferase. The result is a 5'-5'-triphosphate linkage which is characteristic of the mRNA cap structure (Bisaillon and Lemay, 1997). The hepatitis E virus capping enzyme P110, shows similarities to the methyltransferase and guanylyltransferase of alphavirus nsP1, tobacco mosaic virus P126, brome mosaic virus replicase protein 1a and bamboo mosaic virus nonstructural protein. These similarities in properties suggest a common evolutionary origin for the above plant and animal virus families (Magden *et al*., 2001). These authors further propose methyltransferase and guanylyltransferase reactions as potential antiviral drug development targets as both reactions are strictly virus specific.

RNA viruses are replicated in infected cells by viral RNA-dependent RNA polymerases, referred to as replicases (Ishihama and Barbier, 1994; Kanjanahaluethai and Baker, 2000). As well as polymerase activity, the replicase enzyme of influenza virus has a proofreading function (Ishihama *et al*., 1986).

A number of viruses have either a multifunctional enzyme (NS3 of HCV) or several proteins co-localize at the site of viral replication (mouse hepatitis virus - MHV). NS3 of hepatitis C virus (HCV), is a multifunctional enzyme with three known catalytic activities separated into two independent domains. Serine protease activity is located at the N-terminal end of the protein while NTPase and helicase activities are found in the C-

terminal region. It has been further suggested that a protein expressed immediately downstream of NS3 (NS4A) in the viral polyprotein, may play an integral structural role in the NS3 protease domain. There is some evidence suggesting that the domains of NS3 may influence the activity of one another and that interactions between the genomic elements of HCV may regulate the enzymatic activities of this HCV replicase component (Morgenstern *et al.*, 1997).

Proteolytic processing of the replicase gene product of MHV has been shown to be necessary for viral replication (Teng *et al*., 1999). The replicase gene (gene 1) is translated into two polyproteins that are processed to generate in excess of 15 mature proteins. Several of these proteins have been demonstrated to localize at the site of viral RNA synthesis in tightly associated membrane populations (Sims *et al*., 2000). It has further been demonstrated that complexes which are known to be involved in RNA synthesis contain several gene 1 products closely associated with structural proteins located at sites of virion assembly (Bost *et al*., 2000).

In the case of Dengue virus, a mosquito-borne flavivirus, replication occurs at the membrane associated replicase complex (RC). The RC complex includes a number of proteins which are derived from the viral polyprotein. Amongst these are NS3, protease/ NTPase / helicase and NS5 the methyltransferase / replicase. It would seem that the RC may be viewed as a “protein machine” with important protein-protein and RNA-protein interactions determining its replication function. Amongst these interactions, it has recently been demonstrated that the C-terminal region of NS3 interacts with the N-terminal region of NS5 (Johansson *et al*., 2001).

1.10.3 REPLICATION AND TRANSCRIPTION IN BTV

In the case of BTV, virus replication and transcription occur in the virus inclusion bodies (VIBs). The proteins responsible for replication and transcription are the three minor core proteins. VP1 has been identified as the replicase (Urakawa *et al*., 1989; Boyce *et al*., 2004). The role of capping of the mRNA has been ascribed to VP4 (Ramadevi *et al*., 1998a). VP6 has been identified as the helicase with associated NTPase activity (Stäuber *et al*., 1997). An important characteristic relating to helicase function is the ability to bind nucleic acids. BTV VP6 has been identified as a nucleic acid binding protein (Roy *et al*., 1990; Hayama and Li, 1994). As yet, the activity of each of these proteins has been examined independently and their association has not been demonstrated. Very little is known about the corresponding proteins in AHSV. Sequence analysis has suggested similarities in function.

1.11 AIMS

When this study was initiated, very little information was available with regards to the VP6 gene of AHSV or its product. This study focused on the characterization of the structure and function of AHSV VP6. These long term, broad-based objectives were met by addressing the following short term aims:

a) To determine the primary structure of the genome segment encoding VP6 and the VP6 protein in order to identify structural elements or sequence homologies that will assist us in identifying the function of VP6.

b) To characterize the VP6 protein of AHSV with a particular focus on optimizing the expression of the protein and investigating protein features such as solubility, post-translational modification and in particular nucleic acid binding activity.

Both the above aims will enable us to predict a possible helicase function for VP6 of AHSV.

CHAPTER 2:CLONING AND CHARACTERIZATION OF THE GENOME SEGMENT ENCODING VP6 OF AHSV

2.1 INTRODUCTION

Very little is known about the three minor structural proteins of AHSV. Prior to this study, only partial clones of the AHSV VP6 gene were available which provided some 5' and 3' sequence data. Unexpectedly, it was found to be extremely difficult to obtain full-length VP6 gene clones (Cormack, 1996).

Variation at nucleotide level provides the most direct quantitative measurement of the differences between related viruses. It is, therefore, useful to compare the sequence data of closely related viruses such as BTV (the prototype virus of the *Orbivirus* genus) and AHSV as well as viruses within the same serogroup. A comparison of the gene sequence is valuable for the identification of regions of importance that may have been conserved between different orbiviruses or indicate specific regions of interest that are unique to AHSV VP6. In BTV VP6 notable primary structural features include an unusually high glycine content and the highest number of charged amino acids of all the BTV proteins (Fukusho *et al*., 1989). BTV VP6 has nucleic acid binding characteristics and some motifs have been proposed (Roy *et al*, 1990, Hayama and Li, 1994; Kar and Roy, 2003). In 1997, Stäuber *et al*. demonstrated the ATPase and helicase activities of BTV VP6. They reported little similarity to other helicases but some similarity to common helicase motifs. Kar and Roy (2003), identified three motifs consistent with SF2 helicases namely, the Walker A and B motifs and an RNA binding motif similar to a conserved helicase motif. The genome nucleotide sequences of two other orbiviruses have been published; Chuzan virus which is a member of the Palyam serogroup (Yamakawa *et al*., 1999) and the tick borne St Croix River virus (Attoui *et al*., 2001). The availability of this data will facilitate analysis of the sequence of AHSV VP6.

Numerous conserved motifs relating to nucleic acid binding, NTP binding and hydrolysis and helicase activity have been identified. The identification of such motifs are used to make predictions about the functions of proteins.

Eight protein families recognized by common RNA binding motifs have been identified namely: the **RNP** (or otherwise known as the RNA recognition motif - RRM; the arginine-rich motif **(ARM)**; the **RGG** box; the **KH** motif; the **double-stranded RNA binding motif**; the **Zinc finger-knuckle**; the cold shock domain (**CSD)** and finally, the **GRPs** (Glycine-rich proteins) (Mattaj, 1993; Burd and Dreyfuss; 1994; Graumann and Marahiel, 1996; Sachetto-Martins *et al*., 2000).

Another motif important to helicase type proteins is the NTP binding motif. This motif is common to all the helicase superfamilies defined on the basis of a series of conserved motifs (Kadaré and Haenni, 1997).

Prior to the initiation of this study, no information about AHSV VP6 sequence was available because the gene had never been cloned. The aim of the work described in this chapter is to characterize the primary structure of the genome segment encoding VP6 of AHSV. In order to achieve this, the genome segment encoding VP6 of both serotypes 3 and 6 were cloned and sequenced. The resultant nucleotide and amino acid sequences were analyzed.

2.2 MATERIALS AND METHODS

2.2.1 PREPARATION OF dsRNA FOR cDNA SYNTHESIS

cDNA was produced using the cloning method of Cashdollar (1982) with modifications described by Bremer *et al*. (1990). DsRNA of AHSV-9 was purified as described by Van Staden *et al*. (1995) and further purified on sucrose gradients. The gradients consisted of 5 steps from 5 – 40% sucrose made up in 1 x TE (10mM Tris pH 7.4, 1mM EDTA pH 8.0) in 0.1% DEPC (BDH) water. It was centrifuged for 16hrs at 38 000rpm. Sixteen fractions of 10 drops each were collected from the bottom of the gradient using a gradient tube fractionator (Hoefer Scientific Instruments). 2μl of fractions 3 - 14 were run on a 1% TAE agarose gel in the presence of EtBr (Roche). Fractions containing segments 4 – 8 were pooled and precipitated by the addition of NaAc pH 7 to the final concentration of 0.3M and 2 volumes of 96% ethanol. Precipitated dsRNA was resuspended in DEPC UHQ to the final concentration of 1μg/μl as determined by optical density measurements at 260nm in a Du-64 Beckman spectrophotometer.

For polyadenylation of the dsRNA, a mix consisting of 10μg of purified dsRNA diluted in DEPC UHQ to a final volume of 32μl was prepared. In another tube the polyadenylation mix was assembled with the following final concentrations: 60mM Tris pH 8.0; 10mM $MgCl_2$; 3mM $MnCl_2$; 300mM NaCl; 0.3mM ATP (Gibco BRL). 43μl of the polyadenylation mix was added to a tube containing 10μCi of lyophilized ^{3}H-ATP (Amersham). After the addition of the dsRNA mix, the polyadenylation reaction was incubated at 37°C for 50min. The polyadenylated dsRNA was separated from unincorporated free nucleotides by G-75 Sephadex column chromatography.

2.2.2 SEPHADEX COLUMN CHROMATOGRAPHY

Sephadex G-75 (Sigma) swollen overnight in 0.1% DEPC UHQ was packed into a glass Pasteur pipette with a glass bead blocking the mouth of the pipette and allowed to settle. The column was rinsed with 3ml of 1mM Tris pH 8.0. The polyadenylation reaction was layered onto the column and washed in with 600μl of Tris buffer. 12 fractions of 100μl each were collected in separate Eppendorf tubes. 2μl of each fraction was counted in 2.5ml Ready Value™ liquid scintillation cocktail in a Beckman LS 3801 scintillation counter. The peak fractions were pooled in a corex tube and lyophilized. The lyophilized polyadenylated dsRNA was resuspended in 20μl of DEPC UHQ with a concentration of 0.75μg/μl.

2.2.3 cDNA SYNTHESIS

Three μg of polyadenylated dsRNA was denatured by adding 100mM MMOH (methyl mercuric hydroxide) to a final concentration of 20mM and incubating at RT for 30min. Standard cDNA mix A was prepared to the following final concentrations in 1% DEPC UHQ: 64mM Tris pH 8.3; 13mM $MgCl_2$; 150mM KCl; 0.6mM dNTP mix (Roche) and 4mM β-mercaptoethanol incubated at

RT for 30min. Standard mix B was prepared as follows: 47μl of mix A; 20μCi α^{32}P-dCTP; 120U HPRI (human placental RNase inhibitor) (Amersham); 16U AMV reverse transcriptase (Promega), 1μg Oligo dT (Roche) and the denatured polyadenylated dsRNA. The reaction was incubated at 42°C for 60min. The reaction volume was increased to 100μl and passed through a Sephadex G-75 column to separate the cDNA from unincorporated free nucleotides (as described 2.2.2). The fractions were counted in a Beckman LS3801 scintillation counter and peak fractions were pooled. The pooled fractions were lyophilized and resuspended in 20μl of 10 x alkaline buffer (300mM NaOH, 20mM EDTA) and 5μl tracking dye (40% sucrose in alkaline buffer with Blue/Orange loading dye (Promega).

2.2.4 ALKALINE AGAROSE GEL ELECTROPHORESIS

For the purposes of this investigation, specific cDNA segments namely segments 7 – 9 which run together on an agarose gel were required. cDNA was visualized by electrophoresing the above sample in a vertical agarose gel under denaturing conditions. A 1.5% agarose gel was prepared in 1 x Alkaline buffer (30mM NaOH; 2mM EDTA). The sample was loaded on the gel and electrophoresed for 4hrs at 40V, 105mA in a SE 400 Hoefer Scientific Instruments gel electrophoresis unit. Following “wet” gel autoradiography, the band containing segments 7 – 9 was excised from the gel.

2.2.5 GLASSMILK PURIFICATION

The cDNA segments were purified from the agarose by the Glassmilk (BIO 101) purification procedure. The manufacturers protocol was followed with some modifications. Briefly, the excised agarose band was melted in 3 volumes of 6M NaI at 50°C until completely dissolved. 5μl of glassmilk was added and the reaction was incubated at RT with shaking for 15min. The glassmilk-cDNA pellet was collected by centrifugation in a benchtop microcentrifuge at 15 000rpm for 1min. The pellet was washed 3 times in NEW (NaCl, ethanol and water) wash and eluted twice for 10min at 50°C in UHQ.

2.2.6 PREPARATION AND TRANSFORMATION OF COMPETENT *E. COLI* CELLS

Bacterial cells were made competent by means of the $CaCl_2$ method (Mandel and Higa, 1970; Cohen *et al*., 1972). An overnight culture of *E. coli* cells was prepared by inoculating 3ml of LB-broth (1% w/v bacto-tryptone, 0.5% w/v bacto-yeast extract, 1% NaCl pH 7.4) with a single colony and incubating for 16hrs at 37°C with shaking. 100ml of LB-broth was inoculated with 1ml overnight culture and incubated until log phase (OD_{550} approximately 0.5). 20ml of cells were collected by centrifugation in a Beckman JS-7.5 swing bucket rotor at 4000rpm for 3min at 4°C. Cells were resuspended in 10ml ice cold 50mM $CaCl_2$ solution and incubated on ice for 30min. Cells were collected as before, resuspended in 1ml ice cold 50mM $CaCl_2$ solution and incubated on ice for 1hr before use.

Fifteen μl of annealing mix was added to 200μl of cells in a glass test tube. A test transformation with supercoiled pBR322 was also performed. Transformations were incubated on ice for 30min followed by heat-shock for 90sec at 42°C. After 2min cooling on ice, LB-broth was added to a final volume of 1ml and incubated for 1hr at 37°C with shaking. Transformation mixes were plated out on LB-agar plates containing 12.5μg/ml tetracycline (Roche).

2.2.7 PLASMID ISOLATION

Plasmid DNA was isolated using the alkaline lysis method developed by Birnboim and Doly (1979). Large scale isolations were modified according to the manufacturers protocol for nucleobond plasmid isolation (Macherey-Nagel). Cells grown in overnight culture were collected by centrifugation in a benchtop microcentrifuge (miniprep) or a Beckman J2-21 floor centrifuge (large scale) at 5000 rpm, depending on the volume of cells. Volumes for large scale plasmid isolation are indicated in brackets. The pellet was drained and resuspended in 100μl (4ml) solution I (50mM glucose; 10mM EDTA and 25mM Tris pH 8). Cells were lysed by the addition of 200μl (4ml) alkaline-SDS buffer (0.2N NaOH, 1% SDS) followed by incubation for 5 min on ice. 150μl cold 3M NaAc pH 4.8 (4ml 2.5M KAc pH 5.2) was added and incubated for 5min on ice. Chromosomal DNA and SDS precipitated proteins were removed by centrifugation for 10 min (45 min) at 15 000 – 16 000 rpm. The plasmid DNA in the clear supernatant was precipitated by the addition of 2 volumes of 96% ethanol or 0.7 volumes of isopropanol. In the miniprep, the precipitated DNA pellet collected by centrifugation was washed with 80% ethanol, air-dried and resuspended in 20μl of UHQ. The large scale was completed in one of two ways. For nucleobond purification, the supernatant was passed through a nucleobond AX100 column (as per manufacturers recommendations). The DNA trapped in the column was washed and eluted. The eluted DNA was precipitated by the addition of 0.7 volumes of isopropanol followed by a washing step with 80% ethanol. Alternatively, subsequent to precipitation, the pellet was resuspended in 6ml 1 x TE and the RNA was precipitated by the addition of 5ml 7.5M ammonium acetate with incubation for 1hr on ice. The RNA was removed by centrifugation and the plasmid DNA in the supernatant precipitated by the addition of 2 volumes of 96% ethanol. Contaminating protein was removed by phenol / chloroform extraction. The plasmid solution was made up to a volume of 400μl by the addition of 1 x TE. 200 μl of Tris buffered phenol (pH 7.8) and 200 μl of chloroform. The solution was mixed and incubated at RT for 2min followed by centrifugation in a microcentrifuge for 2min. The aqueous phase was transferred to another tube and treated with 200μl of chloroform to remove residual phenol. The centrifuge procedure was repeated and the DNA in the aqueous phase was precipitated by the addition of 2 volumes of 96% ethanol in the presence of 0.3M NaAc pH 7. Following an 80% ethanol washing step, the purified plasmid DNA was dried and resuspended in 300μl UHQ.

2.2.8 PREPARATION OF RECOMBINANT PLASMID DNA BY CESIUM CHLORIDE GRADIENT PURIFICATION

Closed circular plasmid DNA may be separated from contaminants by buoyant density centrifugation in CsCl-ethidium bromide gradients developed by Clewell and Helinski (1969). The bouyant density of most dsDNA in CsCl is 1.70g/cm^3. RNA has a bouyant density of 1.80g/cm^3 and forms a pellet at the bottom of the CsCl gradient while protein (bouyant density of 1.30g/cm^3 in CsCl) usually floats on top (Sambrook and Russell, 2001). 1.617g/ml solutions of CsCl were made up in 1 x TE buffer in 5ml Beckman ultra-clear tubes. The plasmid DNA in a volume of 1ml and 3mg from a 10mg/ml EtBr solution was added to the tubes. The gradients were centrifuged for 40hr at 38 000rpm in a SW50 rotor in a Beckman ultracentrifuge. Under UV light the bottom band (of two, top band was linear plasmid DNA) representing supercoiled plasmid DNA was removed using a bent needle and syringe. EtBr was removed by several washes with UHQ saturated n-buthanol. The washed supernatant was diluted with 1 x TE (to prevent CsCl crystal precipitation) and plasmid DNA was precipitated with 2 volumes of 96% ethanol followed by an 80% ethanol wash. The dried pellet was resuspended in 300µl of UHQ. Concentration and purity were determined using optical density measurements at 260 and 280nm respectively.

2.2.9 AMPLIFICATION BY PCR

5' and 3' sequence data were available for the genome segment encoding VP6 of AHSV-3 and these were used for the design of primers (Cormack, 1996). *Bam*HI sites were incorporated on the ends of the primers to facilitate cloning into expression vectors. Primers were designed with similar melting temperatures calculated using the following formula: $T_m = [4(G+C) + 2(A+T)] – 5$. Three extra bases were added to the 5' ends of the primers to ensure enzyme recognition of the *Bam*HI sites. The sequence of the forward primer was: **VP6.1** 5' *CAG*GGATCCGTTAAATAAGTTGTCTCATGTC 3' and the reverse primer sequence was: **VP6.2** 5' *CAG*GGATCCGTAAGTTTTAAGTTGCCCTC 3'. The PCR reaction was assembled as follows: 100pmol each of primers VP6.1 and VP6.2, 400ng of cDNA, 2.5U TaKaRa Ex Taq and reaction buffer with 2mM $MgCl_2$ and 2.5mM of each dNTP in sodium salts (TaKaRa) according to manufacturers recommendations. The reaction was performed in a Hybaid thermocycler using the following programme: 1 cycle of 95°C for 5min; 51°C for 45 sec; 72°C for 3min followed by 28 cycles of 95°C for 1min; 51°C for 45 sec; 72°C for 3min and completed with 1 cycle of 95°C for 1min; 51°C for 45 sec; 72°C for 5min. Reactions were terminated by incubating on ice. PCR products were separated on a 1% 1 x TAE agarose gel. Using a control of a PCR reaction with constructed VP6 gene as template and molecular weight markers ∅X [PhiX174 / HaeIII] (Promega) and DNA molecular weight marker II (Roche), the amplified VP6 gene was excised from the gel and purified by the glassmilk procedure (2.2.5).

2.2.10 CLONING OF THE PCR PRODUCT

The genome segment encoding VP6 of AHSV-3 and AHSV-6 were cloned into the pBS bluescribe RNA transcription vector (Stratagene). pBS is a 3.2kb colony producing phagemid vector derived from phage f1 and pBS of Stratagene. It has a high copy number with T3 and T7 bacteriophage promoters. Selection of recombinants is facilitated by blue/white selection as a result of α-complementation (Ullmann *et al*., 1967) and ampicillin resistance (Sambrook and Russell, 2001). The PCR products and vector were digested with restriction endonuclease

*Bam*HI. The linearized vector was dephosphorylated using 1U of alkaline phosphatase (Roche) according to manufacturers protocols. Digestion products / dephosphorylation products were purified from contaminating enzymes by the glassmilk procedure. The genome segment encoding VP6 genes were ligated into the prepared pBS bluescribe vector by ligation with T4 DNA ligase (Roche). Bacteriophage T4 DNA ligase catalyzes the formation of phosphodiester bonds between 3'-hydroxyl and 5'-phosphate termini in DNA molecules located adjacent to each other (Weiss *et al*., 1968). Vector and insert were ligated at a ratio of 1 vector : 3 insert in 1 x ligase buffer (66mM Tris-HCl; 1mM DTT; 5mM $MgCl_2$ and 1mM ATP pH 7.5) with 1U of T4 DNA ligase. After 16hrs incubation at 16°C, half of the ligation mixtures were used to transform competent XL1blue cells (Stratagene) made competent using the $CaCl_2$ method described above (2.2.7). Transformation mixes were plated onto plates containing 12.5μg/ml tetracycline (XL1blues have tetracycline resistance) and 100μg/ml ampicillin containing LB-agar plates. Blue/white selection was performed by the addition of the chromogenic substrate X-gal (50μl of a 2% solution per plate) and the lactose analogue IPTG (10μl of a 2% solution per plate) which inactivates the *LacZ* repressor (Sambrook and Russell, 2001). White colonies were selected and incubated as overnight colonies in LB-broth with antibiotics. Putative recombinants were screened after miniprep plasmid DNA extraction on a 1% 1 x TAE agarose gel with pBS as a control. Plasmids with a larger size than the control were digested with *Bam*HI to screen for an insert. Recombinants which yielded the correct insert were digested with *Eco*RI to determine the orientation of the insert. All restriction endonuclease digestion products were analyzed by 1% 1 x TAE agarose gel electrophoresis. Recombinant plasmids containing the genome segment encoding VP6 of AHSV-3 and 6 were selected.

2.2.11 SUBCLONING OF THE GENOME SEGMENT ENCODING VP6

Based on sequence data from AHSV-3 VP6 gene available from Cormack (personal communication), numerous restriction endonuclease digestions were performed. Three sites within the genome segment encoding VP6 namely *Bgl*II, *Pst*I and *Eco*RI were selected for subcloning purposes. The AHSV-3 VP6 gene was subcloned into M13 and single strand sequenced. In order to generate sequence data for characterization of the genome segment encoding VP6 of AHSV including assessment of intra- and interserogroup conservation, the AHSV-6 VP6 gene was subcloned into pBS bluescribe and double strand sequenced. Several internal primers as described by Cormack (1996) were used to overcome difficulties arising from secondary structures.

2.2.11.1 Subcloning into M13

The M13mp range of vectors allows for dsDNA manipulation in the replicative form (RF) and the subsequent generation of ssDNA ideal for manual sequencing. The methods for cloning and manipulation of the M13 vectors were obtained from Sambrook *et al*. (1989). Infection of bacterial cells by M13 bacteriophage requires an intact F pilus. JM109 *E. coli* cells carry an F' episome and will, therefore, support the growth of M13. Genes for enzymes involved in proline biosynthesis have been deleted from the JM109 genome but are supplied by the F' plasmid. In order to maintain the F' plasmid, propagation occurs on medium lacking proline, in this instance M9 minimal medium (1 x M9 salts; 1mM $MgSO_4$; 1mM thiamine HCl; 0.1mM $CaCl_2$ and 0.2% glucose) so that only bacterial cells with the F' plasmid will be proline phototrophs (Sambrook and Russell, 2001).

Large amounts of RF M13mp19 were prepared as follows. An overnight culture of JM109s was

diluted $^{1}/_{40}$ and inoculated with a M13mp19 bacteriphage plaque. After 5hrs incubation at 37°C with shaking, 1ml of the culture was centrifuged for 5min in a benchtop microcentrifuge. 500µl of the supernatant was added to 2.5ml JM109 overnight culture, mixed and incubated at room temperature for 5min. Following dilution to a final volume of 250ml in LB-broth, the cells were incubated for 5hrs with shaking at 37°C. Cells were collected by centrifuging at 4000g for 15min at 4°C. The supernatant could be stored for single-stranded DNA extraction. The pellet was washed in 100ml ice-cold STE buffer (0.1m NaCl; 10mM Tris-HCl pH 8 and 1mM EDTA). Closed circle bacteriophage RF DNA was isolated by the alkaline lysis method. Subcloning proceeded using standard restriction endonuclease and ligation methods.

Competent JM109 cells were prepared as previously described (2.2.7) except that cells were grown to OD_{550} = 0.3 before treatment with $CaCl_2$ commenced. 200µl of competent JM109s were incubated with half the ligation mixes of each subclone of the genome segment encoding VP6 of AHSV-3 and incubated on ice for 40min. The transformation mixes were heat shocked for 75sec at 42°C and cooled on ice. 40µl of a 2% solution of both X-Gal and IPTG were added to 200µl log phase JM109 cells (OD_{550} = 0.3) in a test tube. The competent cells and ligation mixture were added to the test tube together with 3ml of soft agar. The soft agar mix was poured on top of agar plates and incubated at 37°C overnight.

White recombinant plaques were picked using a sterile tip. The plaque was rinsed off into 5ml LB-broth containing 50µl of JM109 overnight culture. This was followed by incubation for 5hrs at 37°C with shaking. 1ml of culture was centrifuged for 5min at RT. SDS was added to a sample of the supernantant to a final concentration of 0.1% and incubated at 65°C for 5min. Recombinant plaques were analyzed by 0.7% 0.5 x TBE agarose gel electrophoresis.

Single-stranded DNA for sequencing was prepared as follows: 1ml of recombinant culture was centrifuged for 5min in a microcentrifuge. DNA was precipitated by treating the supernatant with 200µl of a 20% PEG 6000 and 2.5M NaCl solution. After collecting the pellet by centrifugation, it was resuspended in 1 x TE. Protein contamination was removed by phenol / chloroform extraction as described above followed by ethanol precipitation. After a 70% ethanol wash, the pellet was resuspended in 30µl of UHQ.

2.2.11.2 Subcloning into pBS

The AHSV-6 VP6 gene was subcloned into pBS for double-stranded DNA sequencing. The same restriction enzyme sites were used namely *Bam*HI on the termini, *Bgl*II, *Pst*I and *Eco*RI to generate fragments for subcloning. Recombinant subclones were isolated using the alkaline lysis methods described above (2.2.9) and screened with restriction endonucleases to evaluate the nature of the inserts. Both the coding and non-coding strands were sequenced using VP6.1, VP6.2, M13 forward and reverse primers. The same internal primers as used above were used to sequence through regions with secondary structures. Primers were synthesized by Invitrogen.

2.2.12 MANUAL SEQUENCING

The method of manual sequencing employed was based on the dideoxy chain termination method of DNA sequencing (Sanger *et al*., 1977). DsDNA was denatured to provide a single-stranded template. Sequencing was performed using the Sequenase version 2 kit according to manufacturers protocols (USB). ^{35}S dATP labelled samples were electrophoresed through a 6% denaturing 1 x TBE acrylamide gel. The gel was fixed in a 10% acetic acid, 10% methanol solution and dried on a slab vacuum drier (Hoefer Scientific Instruments) followed by

autoradiography.

2.2.13 SEQUENCE ANALYSIS

Nucleotide sequences were translated using the Microgenie program (Queen and Korn, 1984). Nucleotide alignments were performed using CLUSTAL X 1.81 (Higgins and Sharp, 1988; Higgins and Sharp, 1989; Thompson *et al*., 1997). Amino acid sequences were aligned using CLUSTAL W (Thompson *et al*., 1994) available at the European Bioinformatics Institute. All available different full length orbivirus sequences for the genome segment encoding VP6 / VP6 protein (with the exception of Reovirus λ1 (Dearing strain), due the disparity in length of this putative helicase it was considered inappropriate for inclusion for the purpose of this study) were used in this study and are listed in table 2.1. As two serotypes of the AHSV VP6 gene were used, only two serotypes of BTV were used.

Table 2.1 Full-length sequences for orbivirus VP6 genes / VP6 proteins used in this study.

Virus, Serotype	Protein	GenBank accession number	Reference
AHSV-3	VP6	U19881	Turnbull *et al*., 1996
AHSV-6	VP6	U33000	Turnbull *et al*., 1996
BTV-17	VP6	U55798	de Mattos *et al*., 1996
BTV-10	VP6	D00509	Fukusho *et al*., 1989
Palyam serogroup, Chuzan virus	VP6	AB018088	Yamakawa *et al*., 1999.
St Croix River virus	VP6	AF145406	Attoui *et al*., 2001

2.2.14 PHYLOGENETIC ANALYSIS

Gene trees for the genome segment encoding VP6 / VP6 protein were constructed using PAUP version 4.0b10 (Swofford, 1999). Phylogenetic trees were drawn using an exhaustive maximum parsimony search criterion with consensus topology determined by the 50% majority rule. Statistical support for the trees was calculated using 1000 bootstrap replicates (Felsenstein, 1985).

The VP6 proteins of the viruses used in this study vary in length from 232 to 369 amino acid residues and as a result there are regions of poor alignment. In order to assess the effect of differential indel (insertion – deletion) treatment, all possible options for treatment of indels were considered. The indels were either treated as missing data, in other words ignored, where the tree is based only on fully aligned regions. Alternatively, each site at which an indel occurred was treated as a character with equal weight to any other residue (twenty first amino acid or fifth nucleotide). Finally, an indel (irrespective of size) was treated as a single event where the presence of an gap was treated as a 1 and the absence treated as a 0.

2.2.15 PRIMARY STRUCTURE ANALYSIS

Motif analysis was performed to identify regions that are conserved between different *Orbivirus* VP6 proteins. Analyses were performed using MEME [multiple EM for motif elicitation, motif discovery tool] (Bailey and Elkan, 1994) and MAST [motif alignment and search tool] (Bailey and Gribskov, 1998). Motifs were generated using the eMOTIF maker software (Nevill-Manning *et al*., 1998; Huang and Brutlag, 2001). eMOTIF search software and BLAST (Altschul *et al*., 1990; Altschul *et al*., 1997) were used to determine the significance of the identified motifs. eMOTIF software is available at Stanford University Biochemistry Department, MEME and MAST at the San Diego Supercomputer Center (SDSC) and BLAST at the National Centre for Biotechnology Information (NCBI).

Post-translational modification signals were identified using PROSITE (Bucher and Bairoch, 1994; Hoffmann *et al*., 1999) and protein molecular weight [MW] and isoelectric points [pI] were estimated using Compute pI/Mw (Bjellqvist *et al*., 1993; Bjellqvist *et al*., 1994; Wilkins *et al*., 1998) both available through EXPASY (expert protein analysis system) hosted by the Swiss Institute of Bioinformatics.

2.2.16 HYDROPHILICITY AND SECONDARY STRUCTURE

Hydrophilicity plots were determined according to the method of Kyte and Doolittle (1982) on ANTHEPROT (Deléage *et al*., 1988). The secondary structure of VP6 of AHSV-6 was determined using software available on the 3D-pssm server at the Biomolecular Modelling Laboratory hosted by the Imperial Cancer Research Fund, UK (Fischer *et al*., 1999; Kelley *et al*., 1999; Kelley *et al*., 2000). The 3D-pssm server allows the prediction of secondary structure elements and solvent accessibility of amino acid residues of a query protein. Predictions are made after comparison with master proteins in a fold library. In the library, a three state secondary structure assignment (coil; α-helix and β-sheet / strand) is made per residue using STRIDE (Frischman and Argos, 1995). The secondary structure for the query protein is predicted by PSI-Pred (Jones, 1999). Solvation potential i.e. solvent accessibility is determined by DSSP (Kabsch and Sander, 1983). Solvation potential is defined as the ratio between solvent accessible surface area and overall surface area. Values range from 0% (buried) to 100% (exposed).

2.3 RESULTS

The cloning of full-length VP6 genes was a first priority. Although the traditional procedure for cloning dsRNA genes as described by Cashdollar (1982) with modifications described by Bremer *et al*. (1990) has given satisfactory results for most of the dsRNA genes and in particular the smaller ones, the full-length cloning of the genome segment encoding VP6 remained elusive. In a typical cloning experiment, a large number of full-length copies of the VP7 and NS2 genes would be obtained, but no VP6 genes. This anomaly was investigated once more in an attempt to clone the genome segment encoding VP6 of AHSV by traditional methods.

2.3.1 CLONING OF AHSV SEROTYPE 9

In order to remove all contaminating small RNAs and to enrich the dsRNA in the segment that encodes VP6, the dsRNA isolated from CER (chicken embryo reticulocyte) cells infected with AHSV-9 was purified by two successive fractionations on sucrose gradients. The fractions were analyzed on a 1% TAE agarose gel. Two μl samples of each fraction were electrophoresed and fractions 2 - 5 containing dsRNA were pooled (figure 2.1a and b).

The pooled dsRNA was polyadenylated in the presence of ^{3}H [ATP] (adenine 5' triphosphate) and purified by Sephadex column chromatography. Two microlitre aliquots were counted in scintillation fluid and the peak fractions were pooled. Lyophilized dsRNA was used to synthesize cDNA in the presence of $\alpha^{32}P$ dCTP. The cDNA was purified from unincorporated free nucleotides by Sephadex column chromatography. Samples of each fraction were counted in a liquid scintillation counter. Peak fractions were pooled (figure 2.1c) and lyophilized.

The cDNA was analyzed by vertical agarose gel electrophoresis under denaturing conditions followed by autoradiography (figure 2.2). For the purposes of this study, the cDNA band representing segments 7 – 9 was excised from the gel and purified by the GLASSMILK procedure.

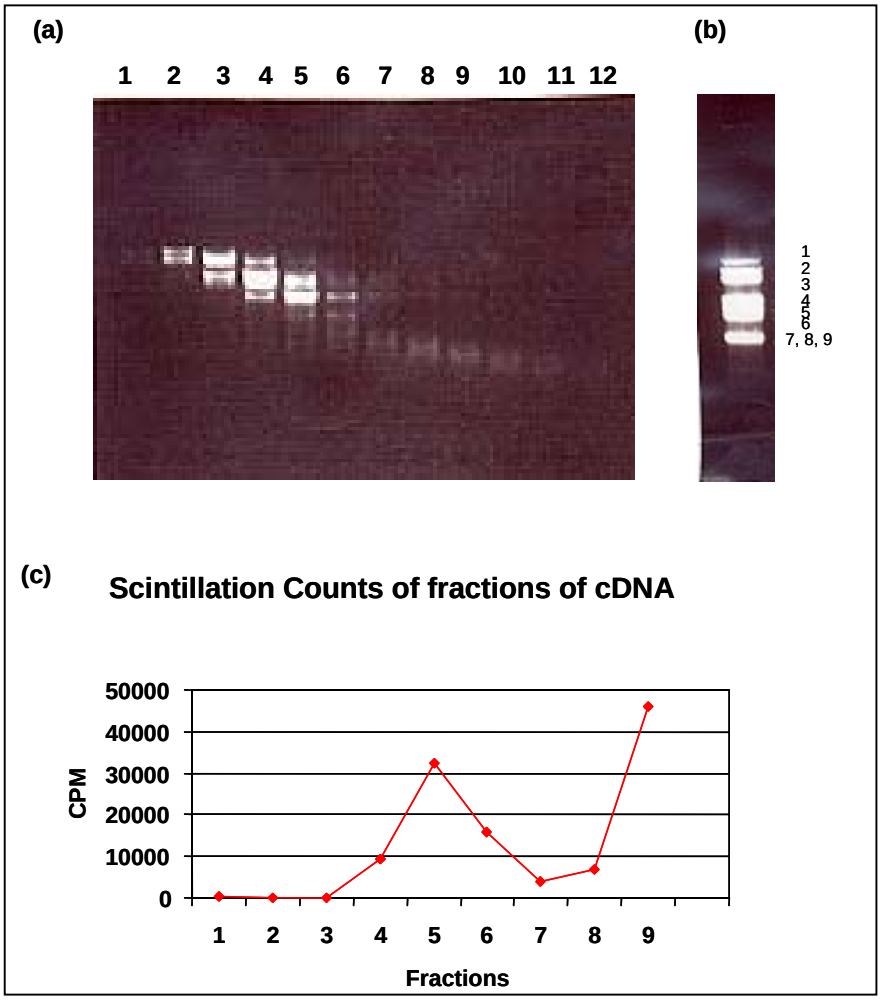


Figure 2.1 AHSV-9 dsRNA analyzed on a 1% TAE agarose gel.

AHSV-9 dsRNA was separated by sucrose gradient fractionation on a 5 – 40% sucrose gradient in a SW50 rotor for 16hrs at 38 000rpm.

(a) Lanes 1 – 12 represent samples of sucrose gradient fractionated dsRNA fractions from the bottom of the gradient (lane 1) to the top of the gradient (lane 12).

(b) Analysis of a pool of fractions (fractions 2 – 5 from 2.1a) which were used for polyadenylation.

(c) Graph depicting the scintillation counts of fractions of cDNA of AHSV-9 labelled with α^{32}P purified by G-75 Sephadex chromatography.

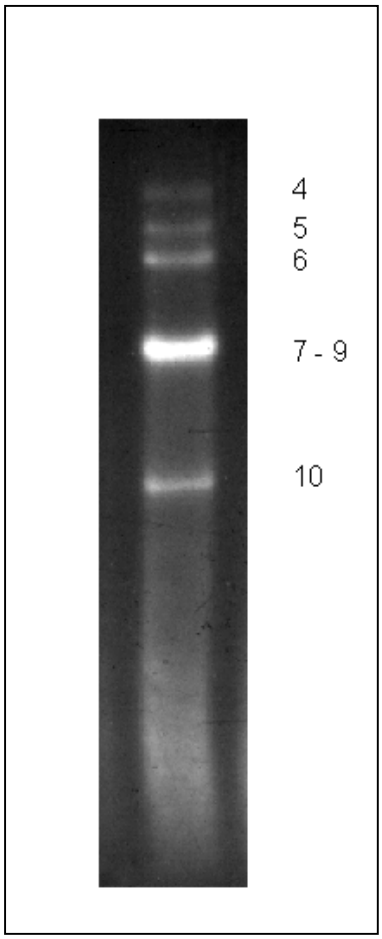


Figure 2.2 Autoradiograph of cDNA synthesized from AHSV-9 polyadenylated dsRNA.

The cDNA was analyzed by 2% alkaline agarose gel electrophoresis. Positions of the genome segments are indicated by the numbers on the right hand side.

2.3.2 AMPLIFICATION OF THE GENOME SEGMENT ENCODING VP6 OF AHSV BY POLYMERASE CHAIN REACTION FROM POOLS OF cDNA

cDNA, prepared from polyadenylated dsRNA of AHSV serotypes 3 and 6 using the method of Cashdollar (1982) with modifications described by Bremer *et al*. (1990), was used as template for a polymerase chain reaction. Primers based on sequence data provided by Cormack (1996) incorporating terminal *Bam*HI sites were used in a PCR reaction (refer to section 2.2.13). The genome segments encoding VP6 of serotypes 3 and 6 were successfully amplified as evidenced by the single amplicons corresponding in size to the 1.3kb DNA marker fragment (figure 2.3). Primer dimmers corresponded in size to the 0.31kb DNA marker fragment. The PCR products were purified from the agarose gel, digested with *Bam*HI and cloned into pBS.

Plasmid DNA from white, putative recombinants was screened against wildtype pBS. Plasmids with a larger size were digested with *Bam*HI (screening for insert) and *Eco*RI (screening for orientation) (figure 2.4). The genome segment encoding VP6 of AHSV-3 in T3 orientation (*Eco*RI digestion yielded a fragment corresponding to the expected size of 291bp) and AHSV-6 in T7 orientation (*Eco*RI digestion yielded a fragment corresponding to the expected size of 878bp) were selected for further use (figure 2.4). Large amounts of plasmid DNA were isolated using a standard large scale method of the alkaline lysis plasmid isolation procedure. Plasmid DNA was further purified by CsCl gradient centrifugation.

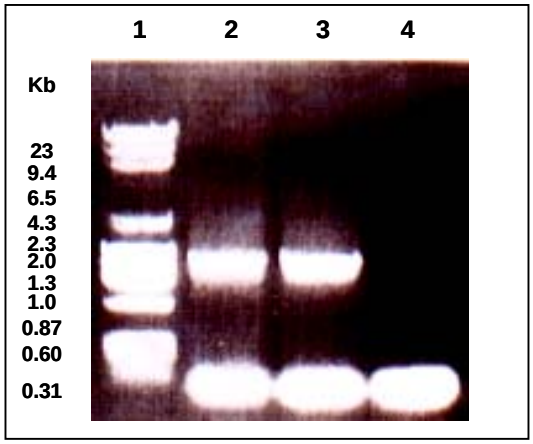


Figure 2.3 PCR amplified VP6 genes from AHSV-3 and AHSV-6 cDNA.

PCR products were separated by 1% TAE agarose gel electrophoresis.

Lanes 1: molecular weight marker.

Lane 2 and 3: PCR products using AHSV-3 and AHSV-6 cDNA as template respectively.

Lane 4: a negative control (no template).

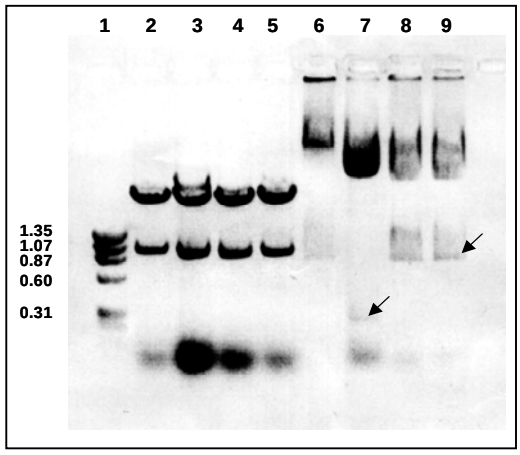


Figure 2.4 Restriction endonuclease selection for recombinant pBS clones.

Restriction endonuclease generated fragments were separated by 1% TAE agarose gel electrophoresis.

Lane 1: molecular weight marker ϕX,

lanes 2 - 3: *Bam*HI digestions of the genome segment encoding VP6 of AHSV-3 in pBS clones.

Lanes 4 - 5: *Bam*HI digestions of the genome segment encoding VP6 of AHSV-6 in pBS.

Lanes 6 - 9: orientation determination of the same clones by *Eco*RI digestions. Arrows show fragments indicating the genome segment encoding VP6 in the T3 orientation (291bp) and T7 orientation (878bp).

2.3.3 SUBCLONING AND SEQUENCING

Sequencing the genome segment encoding VP6 was an important goal as at that time no full-length sequence of the genome segment encoding VP6 of AHSV had been determined. Preliminary RE analysis using *Pst* I, *Bam*HI, *Bgl*II and *Eco*RI indicated that the genome segments encoding VP6 of serotypes 3 and 6 did not contain internal *Bam*HI sites and that this restriction endonuclease could be used to excise the entire gene (result not shown). Following double digestions, a basic restriction endonuclease map was compiled to assist in the development of a subcloning strategy for manual sequencing (figure 2.7). On the basis of these results, the genome segment encoding VP6 of AHSV-3 was subcloned into M13 for single-stranded sequencing and the genome segment encoding VP6 of AHSV-6 was subcloned into pBS bluescribe for double-stranded sequencing. All the subcloned fragments were sequenced using M13 forward and reverse primers. The sequencing results were published and submitted to GenBank (accession numbers: AHSV-3 U19881 and AHSV-6 U33000) (figure 2.5).

Predicted amino acid sequences were generated by Microgenie (Queen and Korn, 1984). The genome segment encoding VP6 of both serotypes was found to be 1169 nucleotides in length with an 1107 nucleotide open reading frame encoding a 369 amino acid polypeptide. The start codon is at nucleotide 18 and the 3' noncoding region is 42 nucleotides in length. The genome segments encoding VP6 of AHSV-3 and AHSV-6 are 96% identical. The conserved sequences GTTAAA at the 5' end and ACTTAC at the 3' end were present (Mertens and Sangar, 1985; Roy, 1989) (figure 2.7).

In order to characterize the AHSV VP6 protein, amino acid compositions of four different orbiviruses were compared in order to determine whether there were significant similarities or differences. Results are shown in table 2.2 with the residue differences between AHSV-3 and AHSV-6 indicated in parenthesis.

AHSV, BTV and Chuzan virus exhibited some similarities in amino acid composition. The most abundant amino acid residue in the VP6 proteins of AHSV, BTV and Chuzan virus is glycine, with AHSV VP6 the most glycine rich. In St Croix River virus VP6 protein, the most abundant amino acid residue is serine which is comparable to the number of serine molecules per thousand found in AHSV VP6. BTV and Chuzan virus VP6 proteins have the largest number of charged amino acid residues, both positive and negative, per thousand residues (363 and 342 respectively). This is followed by AHSV VP6 with in the order of 320 charged amino acids and St Croix River virus with 267. In all four proteins, the number of basic residues exceeds the number of acidic residues. The VP6 proteins have a low cysteine content with AHSV containing four cysteine residues, Chuzan virus has one cysteine residue and BTV and St Croix River virus have no cysteine residues. The most notable characteristics of the amino acid composition of the VP6 proteins are the large number of basic amino acids and the high glycine content. The tick borne St Croix River virus VP6 differs the most of the four in this regard.

```
AHSV-6          GTTAAATAAGTTGTCTCATGTCTTCGGCATTACTCCTTGCACCTGGCGATCTAATCGAAA
AHSV-3          GTTAAATAAGTTGTCTCATGTCTTCGGCATTACTCCTTGCACCTGGCGATCTAATCGAAA
                *******************************************************************

AHSV-6          AAGCAAAGCGCGAGCTCGAGCAACGCTCGATAACTCCGCTTTTGCGGGAGAAAGGTTCGA
AHSV-3          AAGCAAAGCGCGAGCTCGAGCAACGCTCGATAGCTCCGCTCTTGCGGGAGAAAAACTCGA
                ******************************** ******* ************   ****

AHSV-6          AAGAAGCCAAATCTAAATTAAAGGAAGACGGGGAGAAGAAGAACAAGAGTGAAAAGGAAG
AHSV-3          CAGAAGCCAAATCTAAACTAAAAGAGGATGGGGAGAAGAAGAACAAGAGTGAAAAGGAAG
                 **************** **** ** ** ********************************

AHSV-6          AGAACAAAATACATGATGATCGAAGAGTGGAGAGCCAGAAATCTGAGGGAGGCGGACCAG
AHSV-3          AGAACAAAATACATGATGATCGAAGAGTGGAGAGCCAGGAATCTGAGGGAAGCGGATCAG
                ************************************ *********** ***** ***

AHSV-6          CCGATTGTCAACGTGGCGCAGGAAGCGCAGGAGCAAATTGTGCAACATCAACAGGAGGAG
AHSV-3          CCGATTGTCAACGCGGCGCAGGAAGCCGAGGAGCAGATTGCGCAACATCAACAGGAGGAG
                ************* ************  ******* **** ********************

AHSV-6          GAGATGGAAGTGCAGGAGCAAGGACCGGGATTGGAGGGGGAGGAGTGGGAGGAGTGGATT
AHSV-3          GAGATGGAGGTGCAGGAGCAAGGACCGGGATTGGAGGGGGAGGAGTGGGAAGAGTGGATT
                ******** ****************************************** *********

AHSV-6          CGAGATCTGGAGGACATGGAGGACAGGGTGCAGCCTCGGATGGAAAGGGAGTGGGTAAAT
AHSV-3          CGAGATCTGGAGGACGTGGAGGACAGGGTGCAGCCTCGGATGGAAAGGGAGTGGGTAAAT
                *************** ************************************************

AHSV-6          CTAAGACCGGAGCAGATCGTGTCGCTAATGATGATGCAACACGCAATGTTGGTTCCAGTG
AHSV-3          CTAAGACCGGAGCAGATCGTGTCGCTAATGATGGTGCAACACGCGATGTTGGTACCAGTG
                ********************************** ********** ******** ******

AHSV-6          AGGTATCATCTGGTGGAATCACTTCAGGAGGTCTTCAAGGCCGAGGAGGACTCGTTGCAA
AHSV-3          AGGTATCATCTAGTGGAATCACTTCAGGAGGTCTTCAAGGCCGAGGAGGACTCGTTGCAA
                *********** ************************************************

AHSV-6          AGAGTGGTGAATGTGGCGGGGAATCATTGGATAGGATAGGCGGCTGCAGCGGAAATTCAA
AHSV-3          AGAGTAGTGAATGTGGCGGGGAACCATTGGATAGGACAGGCGGCTGCAGCGGAAATTCAA
                ***** ***************** ************ ************************

AHSV-6          AAACTGAGGGAGAGGAGGCGAAGGCTGGAGGGGGCGATAGACGGATTGGAGGATTAGCAA
AHSV-3          AAACTGAGGGAGAGGAGGCAAAGGTTGGAGGGGGCGATAGACGGATTGGAGGATTAGCAA
                ******************* **** ***********************************

AHSV-6          CGCAGGAGATTGCAGACTTTGTGAAGAAGAAGGTCGGAGTTGAAGTTCAGGTGTTTTCTA
AHSV-3          CGCAGGGGATTGCCGACTTTGTGAAGAAGAAGATCGGAGTTGAAGTTCAGGTGTTTTCTA
                ****** ****** ****************** ****************************

AHSV-6          AAGGAATGAGCAACTTATTTACTGTAGATAAGTCATTGCTTGAGCGGGGTGGGTTAGGGA
AHSV-3          AAGGAATGAGTAACTTTTTTACTGTAGATAAATCATTGCTTAAGCGGGGTGGGTTAGGGA
                ********** ***** ************** ********* ******************

AHSV-6          GGGAGGACATTCTACATCAATCAGATATTGTAAAAGAGATTAGAGTAAGTGATAAAAAAG
AHSV-3          GGGAAGACATTCTACATCAATCAGATATTGTAAAAGAGATTAGAGCAAGCGATAAAAAAG
                **** ****************************************** *** **********

AHSV-6          TCAAGATTATTCCTCTTTCCACAGTAAAGAGAATGATAGCGGAATTCGGAGGAACAGAGG
AHSV-3          TCAAGATTATTCCTCTTTCTACAGTGAAGAGAATGATAGCGGAATTCGGAGGAACAGAGG
                ******************* ***** **********************************

AHSV-6          AGGATGAAATCAAAGCTGTTCAAACTCAAAGTTCTTCTATCAGGTATATTAGTAATAGAA
AHSV-3          AGGATGAAATCAAAGCTGTTCAAACTCAAAGCTCTTCTATCAGGTATATTAGTAATAGAA
                ******************************** ****************************
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AHSV-6          TGGAAGATGTTTCAAGAGCGAAGGCGATGTTCACAGCGCCGACGGGTGATGAGGGGTGGA
AHSV-3          TGGAAGATGTTTCAAGAGCGAAGGCGATGTTCACAGCGCCGACAGGTGATGAGGGGTGGA
                **************************************** ***************

AHSV-6          AAGAGGTCGCTAAAGCAGCAACTCAGCGTCCTAACATCATGGCGTATGTGCACGAAGGGG
AHSV-3          AAGAGGTTGCTAAAGCAGCAACTCAGCGTCCTAACATCATGGCGTATGTGCACGAAGGGG
                ******* **************************************************

AHSV-6          AAGGTGATGGATTGAAAGAGCTTTTACATCTGATTGATCATATCTAGGTCCAGGGGTAAA
AHSV-3          AAGGCGATGGATTGAAAGAGCTTTTACATCTGATTGATCATATCTAGGTCCAGGGGTAAA
                **** ****************************************************

AHSV-6          CGGCAGCTTGAGGGCAACTTAAAACTTAC
AHSV-3          CGGCAGCTTGAGGGCAACTTAAAACTTAC
                ****************************
```

Figure 2.5 Nucleotide sequences of the segment encoding VP6 of AHSV-3 (U19881) and AHSV-6 (U33000) aligned using CLUSTAL X.

The start and stop codons are indicated in bold and the conserved 5' and 3' noncoding sequences are underlined.

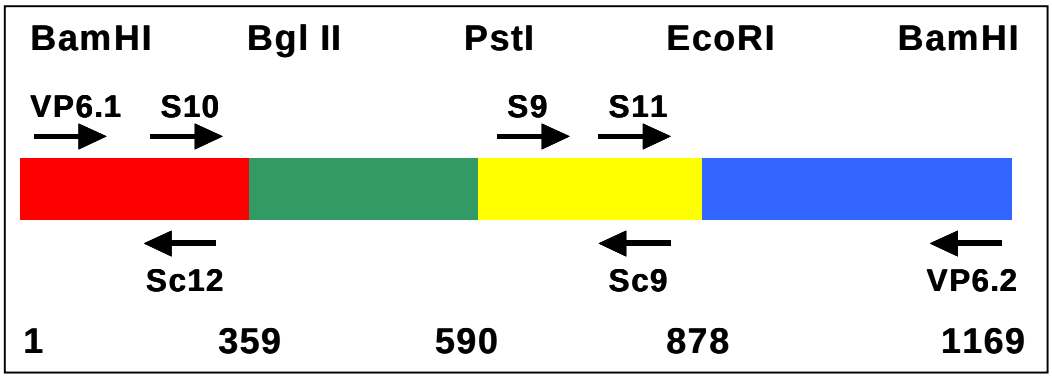

Figure 2.6 Restriction enzyme mapping of the genome segment encoding VP6 of AHSV-3 and -6.

Basic restriction enzyme map of the genome segment encoding VP6 of AHSV-3 and AHSV-6 (including non-coding regions) used for subcloning. Arrows represent the location of internal primers.

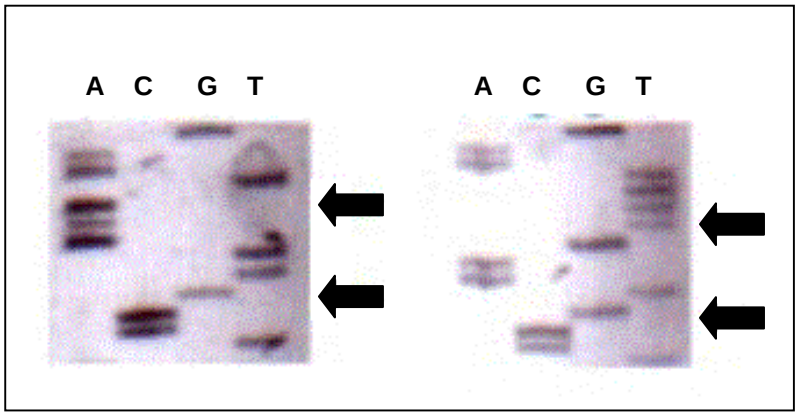

Figure 2.7 Autoradiogram of manual sequencing illustrating conserved 5' and 3' sequences of the genome segment encoding VP6 of AHSV-6.

Table 2.2 Amino acid residue frequencies of AHSV, BTV and Chuzan VP6 expressed per 1000 residues.

Amino acid residue	AHSV-6 VP6 (369)(AHSV-3)		BTV-10 VP6 (328)		CHUZAN VP6 (272)		St Croix VP6 (232)	
	N	/1000	N	/1000	N	/1000	N	/1000
Alanine (A)	30	81	29	88	16	59	19	82
Arginine (R)	22(25)	60 (68)	24	73	14	51	17	73
Asparagine (N)	9 (8)	24 (22)	8	24	8	29	8	34
Aspartic acid (D)	23(24)	62 (65)	19	58	24	88	12	52
Cysteine (C)	4	11	0	0	1	4	0	0
Glutamine (Q)	11	30	11	34	12	44	11	47
Glutamic acid (E)	33(32)	89 (87)	39	119	22	81	17	73
Glycine (G)	62(60)	168 (162)	41	125	30	110	13	56
Histidine (H)	6 (5)	16 (14)	6	18	8	29	5	22
Isoleucine (I)	20	54	19	58	17	63	11	47
Leucine (L)	22(21)	60	16	49	15	55	20	86
Lysine (K)	34(33)	92 (89)	31	95	25	92	11	47
Methionine (M)	6	16	6	18	4	15	6	26
Phenylalanine (F)	5 (6)	14 (16)	2	6	8	29	6	26
Proline (P)	6	16	7	21	3	11	16	69
Serine (S)	35(36)	95 (98)	22	67	21	77	24	103
Threonine (T)	16(18)	43 (49)	19	58	20	74	13	56
Tryptophan (W)	1	3	3	9	1	4	1	4
Tyrosine (Y)	2	5	4	12	5	18	8	35
Valine (V)	22(21)	60	22	67	18	66	14	60
R + K + H (basic)	62(63)	168 (171)	61	186	47	173	33	142
D + E (acidic)	56	151 (152)	58	177	46	169	29	125
F + Y (aromatic)	7 (9)	19 (24)	6	18	13	48	14	60

2.3.4 SEQUENCE ANALYSIS

The most common method of summarizing the relationship between two sequences is by determining their fractional or percentage similarity (Swofford and Olsen, 1990). Both identical and similar (conserved substitutions) were determined from pairwise CLUSTALW alignments (Thompson *et al*., 1994) of the amino acid sequences (table 2.3).

Table 2.3 Amino acid and nucleotide similarity between VP6 of four orbiviruses.

Values are expressed as a percentage of the total number of residues of the longer protein (identical, [similar]). Identity is defined as fully conserved residues. Similarity is fully conserved residues and strong groups which are fully conserved (i.e. conserved substitutions) as determined by CLUSTALW alignment (Thompson *et al*., 1994). Amino acid comparisons are top right of table and nucleotide comparisons are bottom left.

% identical and [similar] amino acid residues (top right of table) and % identical nucleotides (bottom left of table in italics)

	AHSV-6 VP6	AHSV-3 VP6	BTV-10 VP6	BTV-17 VP6	Chuzan VP6	St Croix VP6
AHSV-6 VP6	-	93.5 [96]	27 [46]	26 [45]	24 [42]	12 [29]
AHSV-3 VP6	*96*	-	27 [46]	26.5 [47]	25.5 [40]	12 [28]
BTV-10 VP6	*44*	*47*	-	97 [98]	26 [48]	17 [36]
BTV-17 VP6	*44*	*46*	*98*	-	26 [49]	18 [35]
Chuzan VP6	*37*	*38*	*41*	*41.5*	-	19 [37]
St Croix VP6	*29*	*28.5*	*32*	*32.5*	*37*	-

AHSV shows high interserotype conservation (93.5% identity and 96% similarity on an amino acid level) 96% identity on a nucleotide level. This corresponds to that of BTV amino acid residues showing 97% identity and 98% similarity and 98% nucleotide identity between BTV-10 and BTV-17. AHSV, BTV and Chuzan virus have amino acid identities of between 24 and 27% and similarities ranging from 42 – 49%. There is more variation on nucleotide level between AHSV and Chuzan virus (37 – 38% identity) compared with BTV and Chuzan VP6 (41 – 41.5%). St Croix is the least similar with as little as 12% amino acid identity (29% similarity) to AHSV; 17 - 18% identity (35 - 36% similarity) to BTV VP6 and 19% identity (37% similarity) to Chuzan VP6. Nucleotide identity between St Croix VP6 and the others varies between 29 – 38%. The largest size difference is between St Croix and AHSV VP6 which is reflected in the lowest percentage nucleotide and amino acid identity. Due to the large difference in size of the genes and their encoded proteins, the larger the discrepancy in size, the lower the

percentage identity calculated as number of shared residues divided by the total number of residues of the longer gene or protein.

2.3.5 PHYLOGENETIC ANALYSIS

In 1999, Yamakawa *et al*. determined the nucleotide sequence of the genome of Chuzan virus, a Palyam serogroup virus. They inferred phylogenetic relationships for the different segments of AHSV, Chuzan and BTV using the UPGMA (unweighted pairgroup method using arithmetic averages) method. On the basis of amino acid sequence data analysis, they found that all the Chuzan virus segments were more closely related to AHSV than BTV, with the exception of VP6. In their results, Chuzan virus VP6 grouped with BTV-10 VP6. This result which served as motivation for further homology studies proved problematic for a number of reasons and raised a number of concerns. Firstly, in the UPGMA method, gaps cannot contribute to the phylogenetic resolution of relationships and are excluded from the analysis. Secondly, the UPGMA method assumes a constant rate of evolution (Swofford and Olsen, 1990; Page and Holmes, 1998). This assumption is unlikely to hold true for viruses recovered from different hosts. Thirdly, poor resolution of relationships is likely to occur when gaps are excluded from the analysis where the data set is reduced from 369 amino acids in AHSV VP6 to 232 for St Croix VP6. Conversion of nucleotide data to amino acid sequences results in a three-fold reduction in the size of the data set and is commonly used to determine functional constraints as opposed to evolutionary relatedness. Lastly, there is no obvious reason for the genome segment encoding VP6 of the genome to have a different evolutionary history to the other segments. In the light of these three aspects and in an attempt to confirm or refute the results obtained by Yamakawa *et al*. (1999), both phylogenetic and functional constraint analyses were performed on nucleotide and amino acid sequence data of VP6 respectively.

The evolutionary relationship was determined for four orbiviruses by maximum parsimony methods (PAUP ver 4.0b10) (figure 2.8) using the nucleotide sequences aligned using CLUSTALX (Higgins and Sharp, 1988; Higgins and Sharp, 1989; Thompson *et al*., 1997). Based on the similarities between the *Orbivirus* VP6 genes (table 2.3), St Croix River virus was used as an outgroup. Values for g_1 are used to differentiate between phylogenetic signal and random noise in molecular data sets. If the g_1 values produced for a data set are more negative than the critical values, the data set is considered to be significantly more structured than random data (noise) (Hillis and Huelsenbeck, 1992). A single tree was obtained with a g_1 value of –0.67, $P>0.05$ indicating structured data. BTV and AHSV were found to cluster together. Due to the differences in length of the different VP6 genes, there are regions of poor alignment. A bootstrap value of 100 was obtained when each gap was treated as an individual character; when gaps were treated as individual events and when the gaps were ignored.

The results of Yamakawa *et al*., (1999) suggested that BTV VP6 and Chuzan VP6 were more closely related to one another than to AHSV VP6. In the results obtained in this study one tree was obtained which indicated that AHSV and BTV VP6 genes were more closely related to each other than either were to Chuzan virus VP6.

In order to determine the level of functional relatedness of the VP6 proteins, exhaustive maximum parsimony methods were applied to the amino acid sequences of six orbiviruses (figure 2.9). A single tree was obtained for each analysis, however, the results differed according to how the gaps in the alignments were treated. When the gaps were treated as missing data (i.e. ignored) AHSV VP6 grouped with Chuzan virus VP6 with a bootstrap confidence of 61 (figure 2.9a). Yamakawa *et al*. (1999) obtained this result for all the other virus segments except VP6. When the gaps were treated as single events, the same result was obtained, however, there is no support for the AHSV-Chuzan VP6 branch and as such the tree is considered unresolved (figure 2.9b). Each gap treated as a character generated a tree where AHSV VP6 and BTV VP6 grouped together with a bootstrap value of 74 (figure 2.9c). The four character dataset (a) has a g_1 value of –0.75, P = 0.01 and the binary data set (b) has a g_1 value of –0.79, P = 0.01. These are both relatively negative values indicating significantly more structure to the data that found in random data sets (Hillis and Huelsenbeck, 1992). None of these results correspond to the results obtained by Yamakawa *et al*. (1999).

There are large differences in the number of amino acid residues in the VP6 proteins of different orbiviruses. The number of amino acid residues varies from 369 amino acids in AHSV VP6 to 232 in St Croix River virus VP6 (see figure 2.11 for CLUSTALW amino acid alignment). This suggests that VP6 is not a good choice of gene for phylogenetic analysis and has extremely limited value in functional constraint analyses.

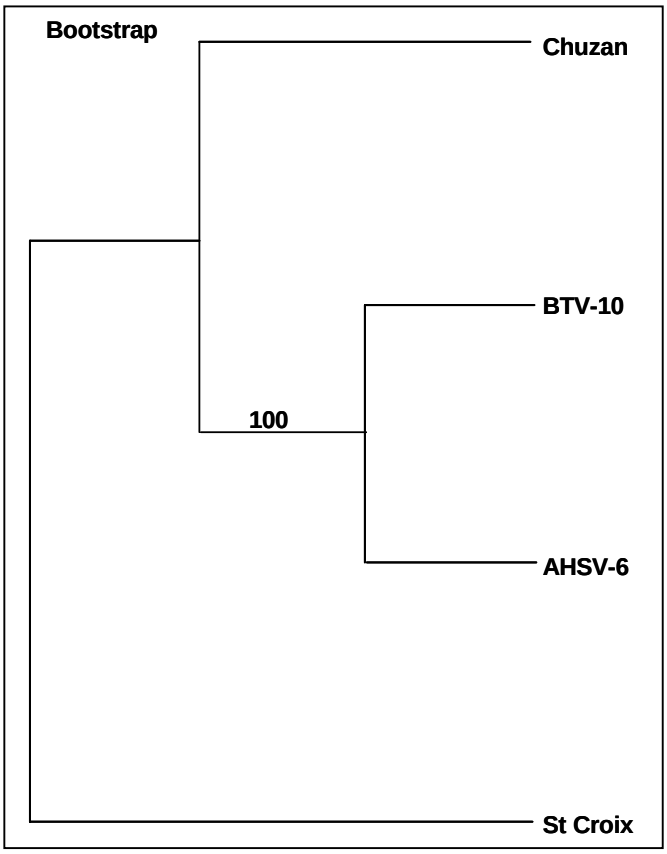

Figure 2.8 Nucleotide phylogenetic analysis of the genome segment encoding VP6 from four *Orbivirus* serogroups.

The phylogenetic tree was reconstructed using exhaustive maximum parsimony methods (PAUP ver 4.0b10).

A bootstrap value of 100 was obtained when each gap was treated as an individual character, when gaps were treated as individual events and when the gaps were treated as missing data (i.e. ignored).

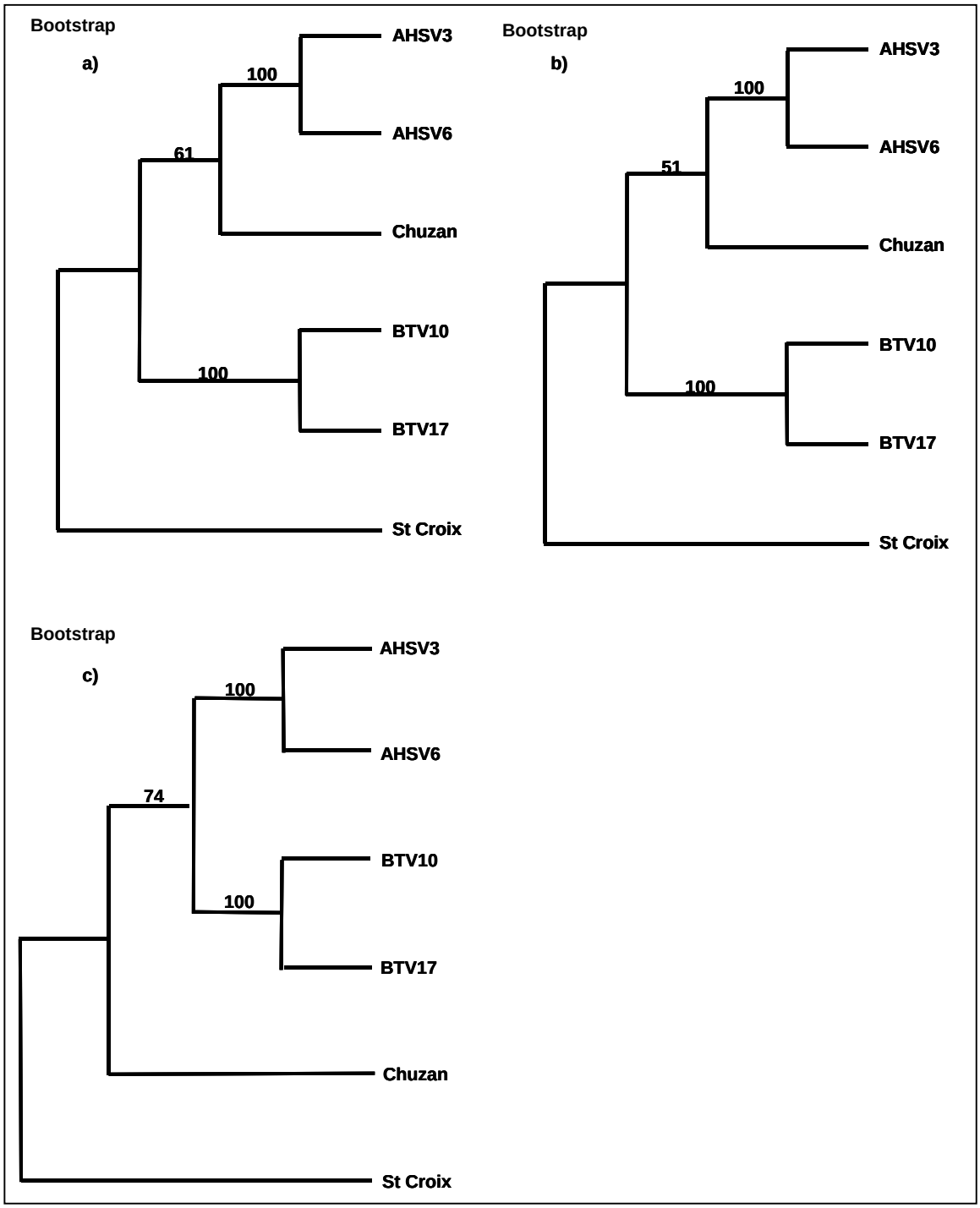

Figure 2.9 Functional constraint analyses of VP6 from four *Orbivirus* serogroups using parsimony methods (PAUP ver 4.0b5).

In a) gaps were treated as missing data (i.e. ignored).

In b) gaps were treated as single events and;

in c) each individual gap was treated as a character.

2.3.6 AMINO ACID SEQUENCE ANALYSIS

In order to identify RNA binding and possible enzymatic activities of AHSV VP6, a search was carried out to identify conserved motifs found in RNA/DNA helicases using a CLUSTALW alignment of VP6 of AHSV-3 and 6 (figure 2.10).

The results of the search are summarized in figures 2.11, 2.12 and table 2.4. In AHSV VP6, a glycine rich region with 36% identity and 45% similarity to glycine rich proteins was identified between amino acids 180 and 192. A region with 91% similarity to the Rep helicase of *E. coli* is also present. Numerous post-translational modification signals were identified. Amongst these are several protein kinase C and Casein kinase II phosphorylation sites and N-myristylation sites (not shown). A N-glycosylation site was also identified located at residues 48 – 51 (figure 2.10a).

VP6 of AHSV shows certain similarities to conserved helicase motifs. Both the A and B sites of the Walker box are present as well as motifs III and IV found in SF2 helicases (figure 2.10b). Molecular weights and isoelectric points were estimated using Calculate pI/Mw (Bjellqvist *et al*., 1993; Bjellqvist *et al*., 1994; Wilkins *et al*., 1998). The predicted molecular weights and isoelectric points of VP6 of AHSV-3 and AHSV-6 were 38 869.28 Da (pI 7.24) and 39 054.46 Da (pI 8.25) respectively.

With the objective of identifying motifs conserved among the VP6 proteins of various orbiviruses, amino acid sequences of AHSV-3 and AHSV-6, BTV-10 and BTV-17, Chuzan and St Croix River virus were aligned using CLUSTALW (Thompson *et al*., 1994). Four motifs were identified using MEME (Bailey and Elkan, 1994) and MAST (Bailey and Gribskov, 1998) (figure 2.11). Motifs were generated using the eMOTIF maker software (Nevill-Manning *et al*., 1998; Huang and Brutlag, 2001). eMOTIF search software and BLAST (Altschul *et al*., 1990; Altschul *et al*., 1997) were used to determine the significance of the identified motifs (table 2.4). Two of the motifs (i and iii) seem to be found in the *Orbivirus* VP6 proteins alone with no other significant similarities detected. Motif ii overlaps with a region with 91% similarity to the Rep helicase of *E. coli*. Similarity to ATP dependent helicases and DNA and RNA directed polymerases were determined. Motif iv showed similarity to ATP binding or dependent transporters and carrier proteins. The three putative RNA binding motifs identified by Hayama and Li (1994) are indicated in figure 2.11. So-called motif 71 overlaps with a motif identified in BTV reported to be important for RNA binding (Kar and Roy, 2003).

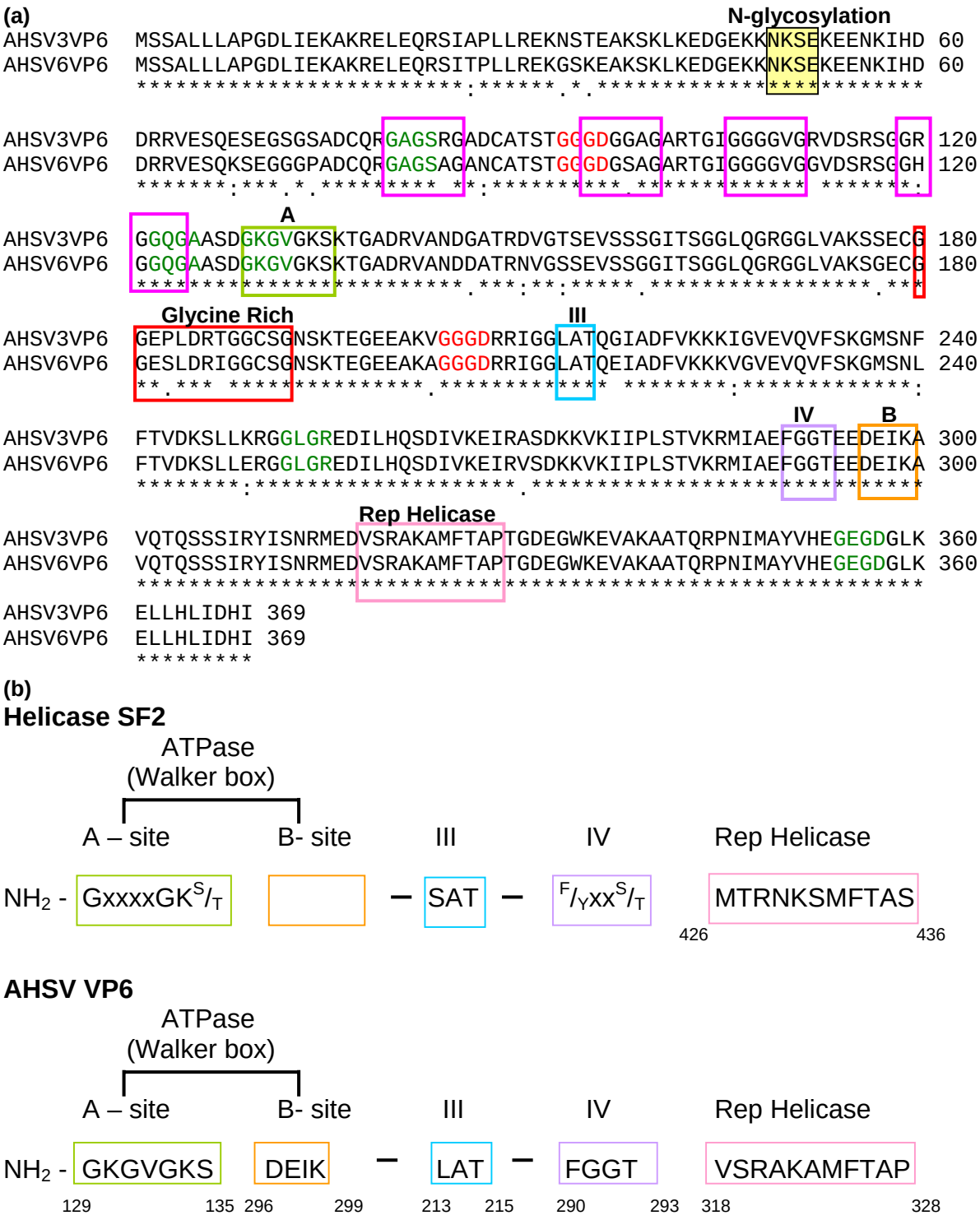

Figure 2.10 (a) CLUSTAL W alignment of VP6 of AHSV-3 and 6.

The yellow box indicates the N-glycosylation site. The red box shows a glycine rich region. (b) Schematic diagram indicating important helicase motifs (Bisaillon *et al*., 1997). The green and orange boxes show the A and B sites of the Walker box respectively. Conserved motifs III and IV found in SF2 helicases are represented by the blue and lavender boxes. The 11 amino acid region with 91% similarity to the Rep helicase of E. coli is indicated by the pink box. Various motifs found in glycine rich proteins are indicated as follows: GxGxxG (pink line box), GGGx (red font) and GxGx (green font).

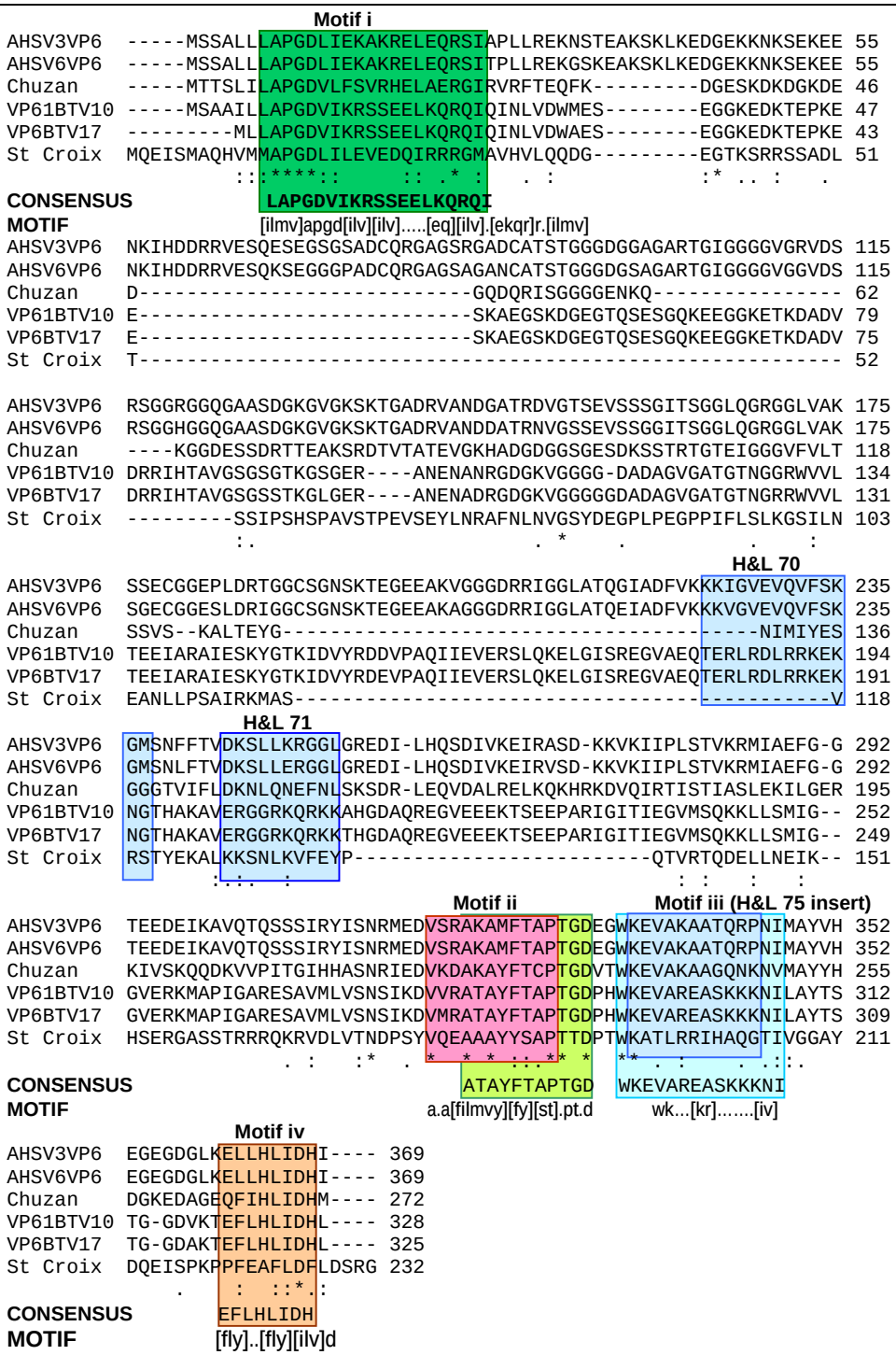

Figure 2.11 CLUSTAL W alignment of VP6 of AHSV-3 and 6; Chuzan virus; BTV-10 and 17 and St Croix River virus.

Four motifs identified using MEME (Bailey and Elkan, 1994) and MAST [motif alignment and search tool] (Bailey and Gribskov, 1998) and eMOTIF maker are indicated by the coloured blocks. Consensus sequences and motifs are indicated below motifs i – iv. The blue blocks are the three amino acid sequences identified by Hayama and Li (1994).

Table 2.4: Motif analysis from VP6 alignment of 6 orbiviruses.

A summary of the motif analysis using *e*MOTIF maker and search software (Nevill-Manning *et al*., 1998; Huang and Brutlag, 2001) and BLAST (Altschul *et al*., 1990; Altschul *et al*., 1997).

MOTIF	SIGNIFICANCE:
Motif i	Found in the *Orbivirus* VP6 proteins analyzed. 88% similarity to a coiled-coil protein. No other significant similarity detected.
Motif ii	Similar motif detected in the following proteins: calcium and potassium transporting ATPases. ATP dependent RNA helicases DDX19 (DEAD box protein), protein kinase P54, ATP dependent DNA helicase PCRA, zinc finger protein REP1, DNA and RNA directed RNA polymerases, Glycoprotein E precursor and RNA helicase MG425/255. The pink block indicates a region of AHSV VP6 with 91% similarity to the Rep helicase of E. coli.
Motif iii	Found in the *Orbivirus* VP6 proteins analyzed. No other significant similarity detected.
Motif iv	Similar motif detected in the following proteins: ATP binding cassette transporter, ATP dependent transporter, ADP/ATP carrier protein 1 precursor (maize), ADP/ATP translocase, potential phospholipid transporting ATPase IA (human) and probable calcium, copper, cation transporting ATPase.
H & L 70 H & L 71 H & L 75	3 motifs identified by Hayama and Li (1994) in BTV VP6, involved in nucleic acid binding activity. H&L 71 putative RNA binding site (Kar and Roy, 2003).

Kar and Roy (2003), proposed that BTV-10 VP6 has motifs consistent with superfamily 2 helicases. These include the Walker A site (common to all NTP hydrolysing proteins), the DEAD/DExH helicase motif and a RNA binding motif. The alignment was expanded to include the other *Orbivirus* VP6 sequences used in this study; to include the full motifs as published by Kadaré and Haenni (1997) for virus-encoded RNA helicases and to examine the importance of the Rep helicase motif (figure 2.12). The ATPase motif is conserved in all the proteins compared. The DEAD/DExH helicase motif (common to SF2 families) is well conserved in the known helicase proteins but not in the *Orbivirus* VP6 proteins. The Rep helicase motif is well conserved in the *Orbivirus* VP6 proteins (with the exception of St Croix River virus VP6). It is not well conserved in the known SF2 helicases. Although the RNA binding motif characteristic of SF2 helicases is well conserved in BTV-10 VP6 and the known SF2 helicases, it is not conserved in any of the other *Orbivirus* VP6 proteins (namely AHSV, Chuzan and St Croix River virus VP6).

Site	ATPase	Helicase	Rep helicase	RNA binding
Motif	**$Gx_{1-4}GK^{S}/_{T}$[1,2]**	**DEAD/DexH**	**[426] MTRNKSMFTAS[436]**	**Tx_5 QRxGRuGR[1] / RxGRxxR[2]**
AHSV-3 VP6	[129] **G**K**G**V**GKS**T[136]	[293] TEE**DE**IKAVQ[102]	[318] VS**RA**K**A**M**FTA**P[328]	[238] SNFFTVDKSLLKRGG[252]
AHSV-6 VP6	[129] **G**K**G**V**GKS**T[136]	[293] TEE**DE**IKAVQ[102]	[318] VS**RA**K**A**M**FTA**P[328]	[238] SNLFTVDKSLLERGG[252]
BTV-10 VP6	[105] NR**G**D**GK**VG[112]	[152] VYR**DE**VPAQI[161]	[278] VV**R**ATAY**FTA**P[288]	[198] **T**HAKAVE**RG**G**R**KQ**R**K[212]
BTV-17 VP6	[102] NR**G**D**GK**VG[109]	[149] VYR**DE**VPAQI[158]	[275] VM**R**ATAY**FTA**P[285]	[195] **T**HAKAVE**RG**G**R**KQ**R**K[209]
Chuzan VP6	[83] ATEV**GK**HA[90]	[42] DGK**DE**DGQDQ[51]	[221] VKDA**K**AY**FT**CP[231]	[140] **T**VIFLDKNLQNEFNL[154]
St Croix VP6	[37] QD**G**E**GT**K**S**[44]	[80] GSY**DE**GPLPE[89]	[177] VQEAAAYYS**A**P[187]	[121] **T**YEKALKKSNLKVFE[135]
HCV1 NS3	[1234]PT**G**S**GKS**T[1241]	[1313]IIC**DECH**SDA[1322]	[2379]DSDAE**S**YSSMP[2389]	[1480]DAVSRT**QR**R**GR**T**GR**G[1494]
DroMLE	[408] NT**G**C**GKT**T[415]	[504] IIV**DEIH**ERD[513]	[1089]IV**R**VDNWLNFD[1099]	[758] SKTNLE**QR**K**GR**A**GR**V[772]
PpvHel	[1255]AV**G**S**GKS**T[1262]	[1339]IIF**DECH**VHD[1348]	[2497]DDF**N**NQFYSLN[2507]	[1513]SYGERI**QR**L**GR**V**GR**[1527]
Reovirus λ1	[6] RKTK**GKS**S[13]	[97] EAK**DEAD**EAT[106]	[978] DLSDMLLEPLL[988]	[421] NFVASCV**R**N**R**V**GR**FD[435]
HuHelA	[412] AT**G**C**GKT**T[419]	[507] VIV**DEIH**ERD[517]	[1080]IVLVDDWIKLQ[1090]	[753] SKTNLE**QR**K**GR**A**GR**S[767]

Figure 2.12 Alignment of *Orbivirus* VP6 putative motifs important for helicase activity with known SF2 helicases.

At the top of the figure are the conserved helicase motifs with their function. Sequences were obtained from GenBank: AHSV-3, U19881; AHSV-6, U33000; BTV-10, D00509; BTV-17, U55798; Chuzan AB018088; St Croix, AF145406; HCV1 (Hepatitis C virus type 1), M62321; DroMLE (*Drosophila melanogaster* maleless protein), M74121; PpvHel (plum pox virus CI protein), M92280; Reovirus λ1 protein, NP-694679 and HuHelA (human RNA helicase), L13848. Motifs were obtained from [1]Kadaré and Haenni (1997) and [2]Kar and Roy (2003).

2.3.7 HYDROPHILICITY AND SECONDARY STRUCTURE

Hydrophilicity plots determined according to the method of Kyte and Doolittle (1982) on ANTHEPROT (Deléage *et al*., 1988) showed that VP6 of AHSV, as in the case of BTV and Chuzan virus, is a predominantly hydrophilic protein (figure 2.13). The hydrophilicity plots of AHSV-3 and AHSV-6 appear to be virtually identical, therefore, only the AHSV-6 hydrophilicity plot is presented here. When the hydrophilicity plots are compared, a large hydrophilic peak is present in all three VP6 proteins at the N-terminal from about redidue 25 – 75. There are several hydrophilic domains similarly distributed throughout the rest of the three VP6 proteins. These results suggest that the proteins are soluble which will facilitate further AHSV VP6 protein related studies.

Since the secondary structure of a protein may be important for the nucleic acid binding function, the secondary structure of VP6 of AHSV-6 was determined using the 3D-pssm server. This analysis allows the prediction of secondary structure and solvent accessibility of each residue of the VP6 proteins (Fischer *et al*., 1999; Kelley *et al*., 1999; Kelley *et al*., 2000) (figure 2.14). The results of this analysis did not reveal secondary structures characteristic of nucleic acid binding proteins. Secondary

structures such as βαββαβ (RNP-1 and RNP-2) and the β5 barrel (CSD) nucleic acid binding proteins were not found in AHSV VP6. Comparisons with two libraries on the 3D-pssm server predicted that AHSV VP6 is a left-handed beta helix. Comparison with another library predicted a beta-roll secondary structure.

Regions of significant solvent accessibility were not identified. This means that specific regions capable of direct contact with nucleic acids cannot be predicted from the amino acid sequence.

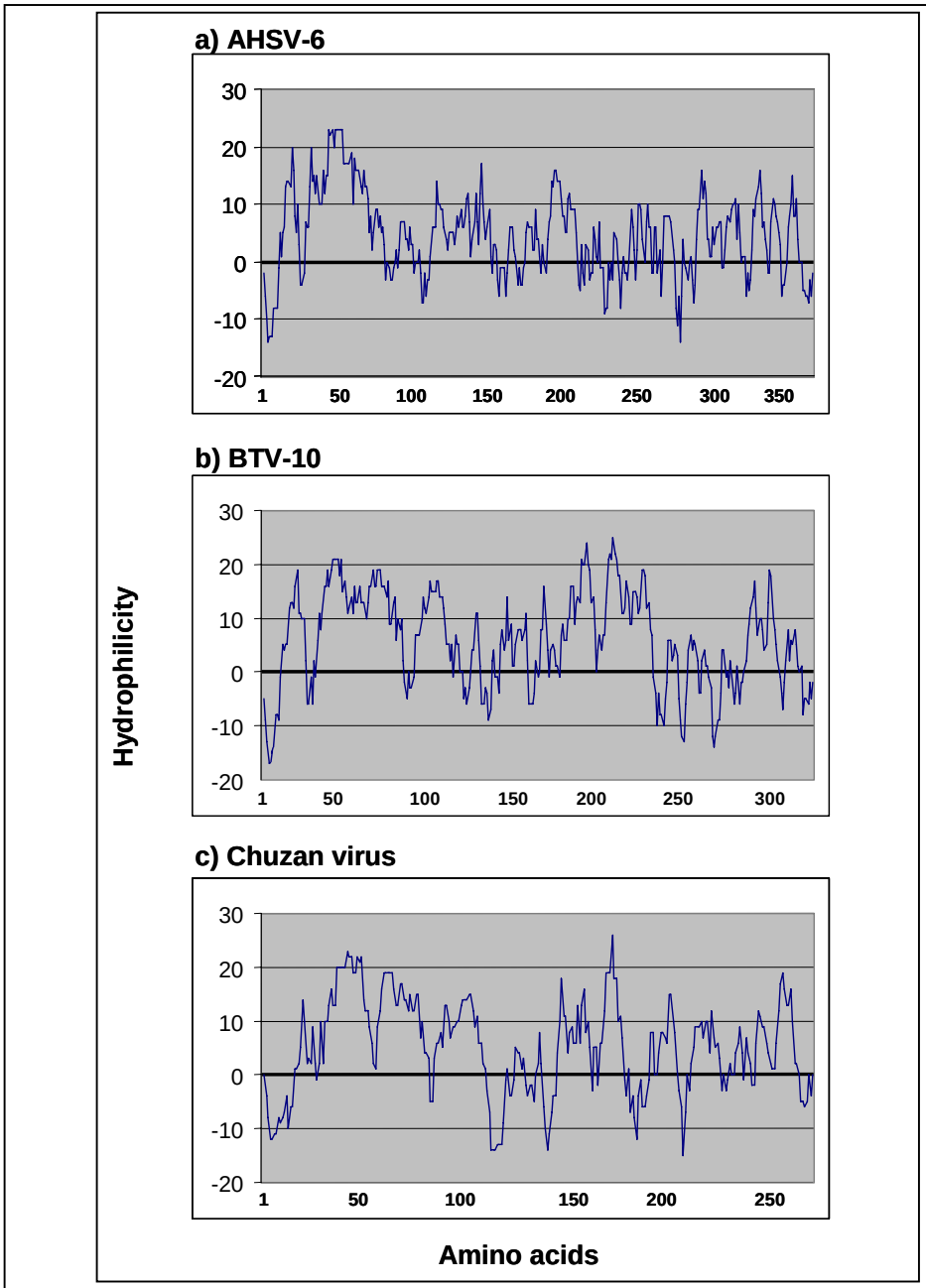

Figure 2.13 Hydrophilicity plots of the VP6 proteins of AHSV-6, BTV-10 and Chuzan virus.

Determined according to the method of Kyte and Doolittle (1982) plotted on ANTHEPROT (Deléage *et al*., 1988).

```
Conf: 95002006406888988866651022244105871343321212312574311013134 1
Pred: CCEEEECCCCHHHHHHHHHHHHHCCCHHHHHCCCHHHHHHHHHCCCCCCCCCCCCCCCC
  AA: MSSALLLAPGDLIEKAKRELEQRSITPLLREKGSKEAKSKLKEDGEKKNKSEKEENKIHD
            10        20        30        40        50        60

Conf: 00012122568881021015567677432245787775433346676662665445678 7
Pred: CCCCCCCCCCCCCCHHHHCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC
  AA: DRRVESQKSEGGGPADCQRGAGSAGANCATSTGGGDGSAGARTGIGGGGVGGVDSRSGGH
            70        80        90       100       110       120

Conf: 88764556664576656632023687542346331477753544423677546467555 5
Pred: CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCEEECCCCCCCCCCCCCCCC
  AA: GGQGAASDGKGVGKSKTGADRVANDDATRNVGSSEVSSGGITSGGLQGRGGLVAKSGECG
            130       140       150       160       170       180

Conf: 88632136667888567632234788860122105889999986419635576078942 8
Pred: CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCEEEECCHHHHHHHHHHHCCEEEEECCCCCEE
  AA: GESLDRIGGCSGNSKTEGEEAKAGGGDRRIGGLATQEIADFVKKKVGVEVQVFSKGMSNL
            190       200       210       220       230       240

Conf: 98702587620999799898999999998735750654210188988863687521235 4
Pred: EEECHHHHHHHCCCHHHHHHHHHHHHHHHHHCCCCEEEEEEHHHHHHHHHCCCCCCCCC
  AA: FTVDKSLLERGGLGREDILHQSDIVKEIRVSDKKVKIIPLSTVKRMIAEFGGTEEDEIKA
            250       260       270       280       290       300

Conf: 55310135761078878864011156788896478899885069988998103765047 8
Pred: CCCCEEEEEEEECCHHHHHHHHEEEECCCCCCCHHHHHHHHCCCCCEEEEECCCCCCHHH
  AA: VQTQSSSIRYISNRMEDVSRAKAMFTAPTGDEGWKEVAKAATQRPNIMAYVHEGEGDGLK
            310       320       330       340       350       360

Conf: 899887059
Pred: HHHHHHCCC
AA:   ELLHLIDHI
```

Figure 2.14 Secondary structure prediction of VP6 of AHSV-6.

This was performed on Psi-Pred (Jones, 1999) using the 3D-PSSM platform at the Biomolecular Modelling Laboratory hosted by the Imperial Cancer Research Fund, UK. The first line (designated Conf:) is the E-value which represents the confidence with which the match is made (0 = low; 9 = high). The second line (designated Pred:) gives the predicted secondary structure class for each residue. C=coil; E=β-strand/sheet and H=α-helix. The third strand gives the amino acid sequence.

2.4 DISCUSSION

The genes encoding the minor core components of AHSV, VP1, VP4 and VP6 have not been characterized in great detail. During the course of this investigation the full-sized VP6 genes of both AHSV-3 and AHSV-6 were cloned and fully sequenced for the first time. Subsequently, a method has been developed for sequence independent amplification of dsRNA segments with a single primer (Potgieter *et al*., 2002) which would have greatly facilitated the cloning of the genome segment encoding VP6.

It was found that both genes are 1169 nucleotides in length with an 1107 nucleotide open reading frame. The genes are flanked by the conserved 5' GTTAAA and 3' ACTTAC sequences (Mertens and Sangar, 1985; Roy, 1989) (figure 2.7). The start codon for both genes is at nucleotide 18 and there is a 42 nucleotide 3' non-coding region. Both polypeptides are 369 residues in length. The sequence results were submitted to GenBank (accession numbers: AHSV-3 U19881 and AHSV-6 U33000).

On a nucleotide level, there is 96% identity between the AHSV-3 and AHSV-6 VP6 genes. The changes are distributed throughout the gene with no one specific region where a cluster of changes occurs. The encoded proteins are highly conserved with 93.5% identity and 96% similarity as determined by CLUSTALW alignment. The predicted molecular weights are in the order of 38.8 - 39kDa. This is slightly larger than the predicted molecular weight for BTV-10 VP6 which is 35 458kDa (Fukusho *et al.*, 1989). Both VP6 of AHSV-3 and AHSV-6 are basic proteins with isoelectric points (pI) of 7.24 and 8.25 respectively.

In order to determine if there were characteristic features of VP6 that are conserved amongst all cognate genes of the orbiviruses, the amino acid content of AHSV-3 and AHSV-6 VP6 proteins was compared to BTV-10 VP6, Chuzan VP6 and St Croix River virus VP6. The number of cysteine residues for all the proteins compared was low. Aromatic residues (F + Y) were low for BTV (18 per 1000 residues) and AHSV (19 / 21). Chuzan and St Croix River virus were higher at 47 and 61 respectively. Glutamine and asparagine content are fairly low for all four orbiviruses. The content of charged amino acids (R + K + H; D + E) is high for all the proteins compared, the lowest being 267/1000 residues for St Croix and highest at 363/1000 residues for BTV. As in BTV VP6 (Roy, 1992), AHSV has a comparatively high number of basic residues. Possibly most significant is the glycine content which for AHSV is 162 – 168 per 1000 residues, 125 for BTV and 110 for Chuzan. For the previous three orbiviruses, glycine is the most abundant amino acid. In St Croix River virus VP6 there are fewer glycine residues (56), the most abundant residue being serine (103). In terms of residue content, AHSV, BTV and Chuzan are most similar and St Croix is somewhat different.

To summarize the relationship between the different VP6 proteins, the percentage similarity was calculated from CLUSTALW alignments for six *Orbivirus* VP6 proteins. Similar to BTV VP6 for which an identity of 97% and a similarity of 98% were observed between serotypes 10 and 17, AHSV VP6 is highly conserved within the serogroup.

Between BTV and AHSV the identity ranges from 26 – 27% and similarity 45 – 47%. When Chuzan VP6 is compared to VP6 of AHSV and BTV the identity falls within the same range (24 – 26%) with a similarity between 40 and 49%. St Croix River virus VP6 is the most distantly related to AHSV with an identity of 12 % (similarity 29%) and closest to Chuzan (19% identity and 37% similarity). It seems evident that the VP6 proteins of AHSV, BTV and Chuzan are most closely related while St Croix River virus is most distantly related. This may be as expected as the first three are biting midge borne orbiviruses while the latter is tick borne (Attoui *et al*., 2001).

Yamakawa *et al*. published the complete nucleotide sequence of the genome of Chuzan virus in 1999. They performed comparative sequence analysis for each segment with BTV and AHSV using UPGMA on amino acid sequence data. UPGMA (unweighted pairgroup method using arithmetic averages) assumes ultrametric data; an equal rate of evolution or an universal molecular clock (Swofford and Olsen, 1990; Page and Holmes, 1998). A broad correlation between the amount of molecular divergence and time is generally accepted. However, it has not been established that rates of evolution are constant. Evidence indicates enough heterogeneity in the rate of evolution that equal rates cannot be assumed (Moritz and Hillis, 1990). Genes do not diverge uniformly in all organisms (or organelles) and as a result systematic errors may well arise should equal rates of evolution be assumed. For each segment, Yamakawa *et al*. (1999) found that AHSV and Chuzan grouped together. The only exception was for the VP6 proteins where BTV and Chuzan grouped together. These results were problematic and as such an attempt was made to formulate a hypothesis of the evolutionary relationship and functional relatedness between the genome segments encoding VP6 of the four orbiviruses above by performing analyses on both nucleotide and amino acid data sets respectively.

Based on the data set of aligned VP6 genes for evolutionary analysis and VP6 amino acid sequences for functional analysis, trees were drawn using maximum parsimony methods performed using PAUP ver 4.0b10 (Swofford, 1999). Parsimony methods gave the best resolution as compared to neighbour joining methods. Characters were weighted to compensate for regions of poor alignment as specified by Swofford and Olsen (1990). All three methods of treating gaps are included in the trees. Bootstrap methods were used to test the robustness of the trees. According to the results with a nucleotide data set, AHSV and BTV grouped together with a bootstrap value of 100. Chuzan grouped on its own and St Croix River virus was used as an outgroup. In terms of inferred common ancestry (nucleotide data) the results presented here indicate that AHSV and BTV VP6 genes are more closely related to each other than to Chuzan virus VP6.

The protein is the most biologically relevant aspect of the gene and may, therefore, be used to infer functional relatedness or constraints (Swofford and Olsen, 1990). It is, however, not considered appropriate for evolutionary hypotheses. It is also implicit that amino acid sequences have only a third of the information of the nucleotide data and as

such fewer informative characters. Yamakawa *et al*. (1999), reported that VP6 of Chuzan virus grouped with BTV-10. Based on their analysis, VP6 of BTV-10 and Chuzan virus are functionally more similar to each other than to AHSV VP6. It was investigated whether parsimony methods would deliver the same results as those of Yamakawa *et al.* (1999) for functional constraints. Accordingly, a functional constraint tree was constructed using amino acid sequence alignments of the VP6 proteins of AHSV-3 and AHSV–6 VP6, BTV-10 and BTV–17, Chuzan and St Croix River virus (outgroup). Three data sets were generated differing in how the gaps were treated. When the gaps were treated as missing data (ignored), AHSV grouped with Chuzan virus with a bootstrap value of 61. In the second data set, the characters were weighted so that gaps in series of more than one were designated 1 = presence of a gap and 0 = absence of a gap i.e. single events, the same tree was obtained but with no support (bootstrap value = 51) and as such the relationship is unresolved. When each individual gap was treated as a character, AHSV grouped with BTV with a bootstrap value of 74. Indels (gaps) arise from biological events which may be as informative as nucleotide or amino acid changes. It would seem that to ignore the gaps would give a skewed picture. However, to treat each individual gap as a character may give inappropriate weight to what may be a single event. To clarify the functional relatedness, more sequence data are required which are currently not available for *Orbivirus* VP6 proteins.

Numerous phylogenetic studies have been performed to determine the relationships between the orbiviruses (De Sá *et al*., 1994; Williams *et al*., 1998; Yamakawa *et al*., 1999; Van Niekerk *et al*., 2001b). The most variable AHSV proteins are VP2 followed by NS3. VP2 is the type specific antigen and NS3 may be useful in differentiating between field isolates and live attenuated vaccine strains of the same serotype (van Niekerk *et al*., 2001b). VP6 proteins appear to be highly conserved within serogroups and very different in terms of total amino acid length between serogroups. VP6, the putative viral helicase, is therefore, not a particularly good choice for determining relationships within or between serogroups.

The amino acid sequences were aligned using CLUSTAL W. The regions of the different VP6 proteins that have the highest number of conserved residues are located in the amino terminal (first fifty residues) and the carboxyl terminal (last 126 residues). The difference in the number of amino acids in the VP6 proteins of the different orbiviruses (AHSV 369 amino acids to St Croix River virus 232 amino acids) is predominantly as a result of stretches of amino acids present in AHSV that are missing from some or all of the other VP6 genes used for comparison (figure 2.11).

RNA viruses evolve very quickly (Worobey and Holmes, 1999). Two types of genetic exchange are found in segmented RNA viruses namely, reassortment and recombination. In the case of recombination, a “donor” nucleotide sequence is inserted into an “acceptor” RNA molecule which results in a new RNA consisting of genetic information from more than one source (Worobey and Holmes, 1999). Homologous recombination has been described in rotaviruses (Suzuki *et al*., 1998). When strict

alignment is not maintained, genetic information may be lost or gained. From the protein alignment of the four *Orbivirus* VP6 proteins, it appears that the difference in length between AHSV, BTV, Chuzan and St Croix River virus VP6 proteins may be explained by a single indels. The amino acid sequences that have been inserted or deleted are most probably nonessential to the function of the protein.

Helicases enzymatically unwind double-stranded nucleic acid molecules by translocating along one strand. Mechanisms proposed for helicase action have two important features. Firstly, ATP-driven conformational rearrangements that provide energy and secondly, nucleic acid binding sites for binding single and double-stranded nucleic acids (Kadaré and Haenni, 1997). Five helicase families have been identified by means of a number of amino acid motifs. All the proteins grouped within these families have ATP binding properties and have the Walker A motif (phosphate-binding loop or P-loop) and the Walker B (Mg^{2+} binding aspartic acid) motifs (Caruthers and McKay, 2002). Superfamilies 1 and 2 have 7 characteristic motifs. SF1 includes alphavirus-like NSP2 proteins and SF2 includes the DexH/DEAD protein family. SF3 proteins have three conserved motifs and include the picornavirus 2C-like proteins (Kadaré and Haenni, 1997). The fourth family includes proteins which are related in sequence to the *E. coli* DnaB protein and describes hexameric ring-like helicases. The fifth family of proteins have sequences similar to the β subunit of proton-translocating ATPases (Caruthers and McKay, 2002). The seven conserved helicase motifs are presented in the table below with an example and functional interactions for each (**Table 2.5**).

Eight nucleic acid binding protein families have been identified on the basis of common conserved sequences. These motifs include the RRM (RNA recognition motif); the ARM (rich in arginine); RGG box (RGG repeats); KH motif; the dsRNA binding motif comprising 9 protein families characterized by a zinc-finger-knuckle; cold shock domain (CSD) consisting of conserved aromatic basic residues and GRPs (glycine rich proteins) characterized by GGGx/GGxxxGG or GxGx motifs.

Table 2.5: Conserved helicase motifs and functional interactions.

Abbreviations are as follows: b is a bulky aliphatic or aromatic hydrophobic amino acid; u is a bulky aliphatic amino acid and x is any amino acid. Functional interactions were determined by crystallographic structures with residues (in bold) involved in the interactions. The table was compiled using information from Kadaré and Haenni (1997)[1] and Caruthers and McKay (2002)[2].

Motif	Classical consensus sequence[1]	Conserved motifs of HCV, SF2 helicase[2] (alternative minimal motifs in parenthesis where applicable)	Functional interaction[2]
I	$b_2x_4GSGxGK^S/_Tx_3bP$	APTGSG**KT** ($GK^T/_S$)	The lysine (K) residue interacts with the phosphates of MgATP/MgADP and the Mg^{2+} ion is ligated by the threonine (T) or serine (S) residue.
Ia	$Rxbu_2xPTRxux_2Eb$	**P**SVAAT	Interacts with single-stranded nucleic acids. The backbone carbonyl of the P residue bonds with the oligonucleotide phosphodiester backbone.
II	b_4DExH	**DE**CH (/DEAD)	The aspartic acid (D) is thought to coordinate interactions with the Mg^{2+} ion of MgATP/MgADP while the glutamic acid (E) supposedly functions as a catalytic base (a Lewis base) during hydrolysis of ATP. H residue interacts with motif VI Q residue.
III	bxuTATPP	**T**AT (/ SAT)	T hydrogen bonds with H of motif II
IV	ub_2uPS	LIFCHS**K**K	K interacts with ssDNA
V	$BubxTDbxExGuxbx_4u_2$	ALMTGFT	No specific interactions documented
VI	$Tx_5QRxGRuGR$	**Q**RRGRTGR (QxxGRxxR)	Q interacts with H of motif II

The amino acid sequence of AHSV VP6 was analyzed to identify any motifs or amino acid signatures that could be used to predict the function of AHSV VP6. In the first instance, the amino acid sequences of the two AHSV serotypes were aligned. The Walker A and putative B motifs; similarity to the conserved helicase motifs III and IV of the SF1 and SF2 helicases and 91% similarity to a sequence found in the Rep helicase were identified. Several motifs characterizing glycine rich proteins were present as well as a N-glycosylation signal. Comparison of the AHSV VP6 amino acid sequence with those of BTV, Chuzan and St Croix River virus VP6 proteins showed four conserved amino acid regions. Two of these were found only in the amino acid sequences evaluated (namely motifs i and iii). Motif i showed similarity to a coiled-coil protein and motif iii was common to the *Orbivirus* VP6 proteins with no other significant similarity.

Motif ii showed marked similarity to ATP dependent helicases and overlapped with the region of high similarity to the Rep helicase. This region may be of functional importance in helicase activity. Motif iv showed similarity to ATP binding or dependent transporters and carrier proteins. No indication of conserved nuclear targeting signals reported for BTV VP6 (Yi *et al*., 1996) was determined from multiple *Orbivirus* protein alignments. Motif iii overlapped with a domain identified by Hayama and Li (1994) in BTV VP6 to be important in nucleic acid binding activity (designated 75). Hayama and Li (1994), describe three epitopes (70, 71 and 75) comprising two binding domains rich in basic residues (arginine and lysine). Motifs designated 70 and 71 were not well conserved in AHSV VP6.

In 2003, Kar and Roy proposed that BTV-10 VP6 has three motifs consistent with SF2 helicases. They presented a sequence alignment comparing BTV-10 VP6 to known SF2 helicases with respect to these motifs namely, the Walker A and B motifs and an RNA binding motif (similar to the conserved helicase motif VI, table 2.5). The alignment presented by Kar and Roy (2003) was expanded to include comparisons to AHSV serotypes 3 and 6 VP6; BTV-17 VP6; Chuzan VP6 and St Croix River virus VP6 proteins. The Rep helicase motif that is conserved within the *Orbivirus* VP6 proteins was included to assess its relevance with regard to other helicases.

The Walker A motif is the most conserved motif across the helicase families. Originally defined as GxxxxGKT, it requires a minimum of GK($^{T}/_{S}$) (Caruthers and McKay, 2002). It is characteristic of NTP dependent nucleic acid translocases (Soultanas and Wigley, 2001; Singleton and Wigley, 2002). Kar and Roy (2003), were able to demonstrate the importance of the lysine (GxG**K**T) in the Walker A motif for ATP binding, hydrolysis and RNA unwinding. This motif is conserved in all the amino acid sequences compared.

Kar and Roy (2003), evaluated the effect of modifying a putative helicase motif DEVP which resembles the DEAD / DExH motif of SF2 helicases. This represents the Walker B motif which was originally defined as a single aspartic acid (D) but most often takes the form of DExx (Caruthers and McKay, 2002). Kar and Roy (2003), changed the glutamic acid (E) to an asparagine (N). RNA binding and unwinding was only slightly affected by this change suggesting that this motif is not important for BTV VP6 enzyme activity. There is more than one DExx sequence in the proteins analysed, possibly this is not the correct one. Although several DExx motifs are present in all the *Orbivirus* VP6 proteins analyzed, the DEAD /DExH motif is not present.

The RNA binding motif identified in BTV VP6 corresponds to Hayama and Li's motif 71. By changing the **R**xGRxxR in the putative helicase RNA binding motif, RNA binding and unwinding as well as ATP hydrolysis activities were significantly reduced (Kar and Roy, 2003). These authors suggest that the functions are interlinked and require the same domains. This motif is highly positively charged and contains a RGG motif in AHSV and BTV VP6 proteins. Three basic amino acids are found in this region in AHSV and may perform a similar function even although the motif *per se* is not conserved.

The Rep helicase motif included in the alignment with known helicases (figure 2.12) is well conserved in AHSV and BTV VP6 proteins, less so in Chuzan VP6 and not conserved in St Croix River virus VP6. It is not conserved in the known SF2 helicases.

There are a number of short motifs characteristic of helicases in the AHSV VP6 protein. These include the Walker A motif, a putative Walker B motif and conserved motifs III and IV. Although not conserved in the SF2 helicases, the Rep helicase motif is conserved in the *Orbivirus* proteins and shows similarity to ATP dependent helicases. As such, it may play a role in *Orbivirus* helicases. However, based on the sequence analyses performed, there are no obvious target motifs for nucleic acid binding activity.

Hydrophilicity plots revealed that VP6 of AHSV-3, AHSV -6, BTV-10 and Chuzan are all predominantly hydrophilic proteins. This suggests that the proteins are soluble. Secondary structure plays and important role in some nucleic acid binding proteins. The RNP-1 and RNP-2 proteins have a βαββαβ secondary structure which forms a four-stranded antiparallel β sheet. As a result, the charged and aromatic side chains of the two sites are solvent exposed and can make direct contact with the bound RNA (Burd and Dreyfuss, 1994). Graumann and Marahiel (1996), report that the secondary structure of CSD proteins, which bind double-stranded and single-stranded DNA as well as RNA, have a β5 barrel secondary structure. GRPs which are proposed to be involved in protein-protein interactions, have a highly flexible secondary structure as a result of their glycine content (Sachetto-Martins *et al*., 2000). Secondary structure predictions were performed using the 3D-pssm server (Fischer *et al*., 1999; Kelley *et al*., 1999; Kelley *et al*., 2000).

The α-helix is a rod like, conformationally stable arrangement which involves a single polypeptide chain. The α-helix may be disrupted by charged side chains which repulse each other electrostatically or by a proline residue which causes a bend in the polypeptide backbone. The β-sheet is a two dimensional arrangement involving one or more polypeptide chains. Parallel or anti-parallel pleated sheets are formed with intrachain hydrogen bonds. The α-helix and β-sheet elements are combined in many ways as a polypeptide folds back on itself in a chain. For steric reasons, glycine is frequently encountered in reverse turns at which the polypeptide changes direction. The single hydrogen of the side chain prevents crowding. Combinations of α-helix and β-sheet elements produce a variety of supersecondary structures (Campbell, 1995). It can be predicted that the AHSV VP6 polypeptide has numerous reverse turns in its secondary structure as a result of the glycine rich residue content.

Fold recognition by comparisons with two libraries on the 3D-pssm server, predicted that VP6 of AHSV-6 is a single-stranded left-handed beta helix as found in the trimeric LpxA-like enzymes belonging to the UDP N-acetylglucosamine acetyltransferase and Xenobiotic acetyltransferase families. Comparison to another library predicted a beta-roll secondary structure as found in metalloprotease families. Solvent accessibility as

determined by DSSP (Kabsch and Sander, 1983) did not reveal regions of significant accessibility.

In a first step towards characterization of AHSV VP6, the primary structure of the genome segment encoding VP6 and encoded protein was analyzed. For further analysis of functional aspects of VP6, expression of the AHSV VP6 genes was investigated. This work is described in the next chapter.

CHAPTER 3:CHARACTERIZATION OF THE VP6 PROTEIN OF AHSV

3.1 INTRODUCTION

The eventual aim of this study was to characterize certain functional aspects of AHSV VP6. Some indications of the possible function were obtained from the analysis of sequence data. Various post-translational modification signals were identified in the amino acid sequence of AHSV-VP6. These include N-glycosylation, protein kinase C phosphorylation, casein kinase II phosphorylation and N-myristylation sites. Post-translational modifications may play an important role in the function of VP6 or the regulation of its activity. In order to study these aspects, it is necessary to express the proteins at sufficiently high levels.

Numerous *in vitro* and *in vivo* systems have been developed for the expression of foreign proteins. An efficient cell-free system for the synthesis of proteins from exogenous RNA templates is the rabbit reticulolysate system. Endogenous mRNA is destroyed with a calcium dependent nuclease. Commercial kits are available which provide a rapid method for determining whether a cloned gene expresses the expected full-length protein. Although expression levels are low, pulse labelling allows visualization of expressed proteins following autoradiography.

Baculoviridae are a family of DNA viruses infectious for holometabolous insects. Initial interest in these viruses was due to their ability to naturally control insect pest populations. They are now widely used as expression vectors for the production of proteins under control of the polyhedrin promoter (Blissard and Rohrmann, 1990).

The BAC-to-BAC baculovirus expression system (Life Technologies) was developed by Luckow *et al*. (1993). The system involves the generation of recombinant baculoviruses by site-specific transposition of a DNA cassette into a baculovirus shuttle vector (bacmid) which is propagated in *E. coli* cells. The gene to be expressed is cloned into the donor plasmid pFASTBAC which has a baculovirus promoter and the left and right ends of a mini *att*Tn7 site which is a target site for bacterial transposition. Transposition occurs in DH10BAC cells which contain a helper plasmid pFASTBAC-GUS which provides transposition functions *in trans*. Recombinant bacmids have a white, kanamycin, gentamycin and tetracycline resistant phenotype. Composite bacmid DNA is isolated from overnight cultures and used to transfect Sf9 cells using a cationic lipid reagent. A series of pFASTBAC Ht vectors have been designed which provide three reading frame options (Hta – Htc). Life Technologies has developed a corresponding vector series pProEx Ht with identical reading frames for expression in bacterial cells. Both the pFastBac Ht and pProEx Ht systems have the same expression cassette. This includes a start codon, a six histidine tag, a Tobacco Etch Virus (TEV) protease

cleavage site, the same multiple cloning site and a stop codon. Frameshift is achieved by an insertion of one or two nucleotides in the *Bam* HI restriction enzyme site. The pFastBac Ht and pProEx Ht systems are useful for comparing expression levels as they allow expression of proteins in insect or bacterial cells that have the same non-viral protein extensions.

The aim of the work described in this chapter is the characterization of the VP6 protein, of AHSV. In order to achieve this aim, the proteins were expressed and various characteristics such as solubility and post-translational modifications were investigated.

3.2 MATERIALS AND METHODS

3.2.1 *IN VITRO* EXPRESSION

T3 RNA polymerase was supplied by Promega and T7 RNA polymerase was supplied by Roche. The genome segment encoding VP6 of both AHSV-3 and AHSV–6 were previously cloned into the expression vector pBS in the T3 and T7 orientations respectively. The templates were prepared by linearization on the 3' end of the multiple cloning site with *Sma*I and *Hind*III. After digestion, the products were purified by phenol / chloroform extraction and analyzed on a 1% 1 x TAE agarose gel. Approximately 1μg of linearized plasmid was used in each transcription reaction. The *in vitro* transcription was performed according to the manufacturers protocols. Transcription efficiency was evaluated on a 0.8% TAE agarose gel made with DEPC UHQ. The equivalent of 1μg of mRNA was used for *in vitro* translation in a rabbit reticulolysate system.

3.2.2 *IN VITRO* TRANSLATION

In vitro translations were performed according to the method described by van Staden and Huismans (1991) following manufacturers protocols. Briefly, mRNA was denatured using 10mM MMOH for 10min at RT (truncated proteins were expressed when mRNA was not denatured prior to translation). Denatured mRNA was translated using the rabbit reticulolysate system (Amersham) in the presence of 30μCi of L-^{35}S-methionine (ICN). A negative control without mRNA was performed. The translation reactions were evaluated by SDS-PAGE. 5μl of *in vitro* translation sample was diluted in 50μl of 2 x PSB and denatured at 95°C for 3min. 10μl of each sample was loaded on the gel. The radioactive label was amplified by soaking the gel in Amplify (Amersham) for 30min and dried on a slab dryer followed by autoradiography.

3.2.3 POLYACRYLAMIDE GEL ELECTROPHORESIS

SDS-PAGE was performed according to the method described by Laemmli (1970). The stacking gel contained 5% of a 30% acrylamide / 0.8% bisacrylamide solution; 0.125M Tris and 0.1% SDS pH 6.8. The separating gel contained either 12% or 15% of a 30% acrylamide / 0.8% bisacrylamide solution; 0.375M Tris, 0.1% SDS pH 8.8. The gels were polymerized by the addition of 10% ammonium persulphate and TEMED. Electrophoresis was performed on a mighty small or a Studier SE 400 vertical gel apparatus (Hoefer Scientific Instruments). The TGS electrophoresis buffer consisted of 0.025M Tris-HCl pH 8.3; 0.192M glycine and 0.1% SDS. Electrophoresis was performed at 120 -130V. Gels were stained in 0.125% Coomassie brilliant blue; 50% methanol and 10% acetic acid and destained in 5% methanol and 5% acetic acid with heating.

3.2.4 *IN VIVO* EXPRESSION USING THE BAC-TO-BAC SYSTEM

The BAC-to-BAC baculovirus expression system (Life Technologies) which was developed by Luckow *et al*. (1993) allows the production of recombinant bacmids with manipulations in *E. coli* cells. The BAC-to-BAC donor plasmid, pFASTBAC has an extensive multiple cloning site. The genome segment encoding VP6 of AHSV-3 and –6 were cloned into the *Bam*HI site. Insertion and orientation were determined by digestion with *Bam*HI and *Eco*RI. Recombinant pFASTBAC molecules were purified using the nucleobond AX100 plasmid purification kit (Macherey-Nagel) as described previously (2.2.9).

3.2.5 PREPARATION OF COMPETENT CELLS BY THE DMSO METHOD

Competent DH10BAC cells were prepared by the DMSO method described by Chung and Miller (1988). The method was the same as described previously for the $CaCl_2$ method until log phase cells were collected (2.2.7). Cells were resuspended in ice cold TSB (1.6% w/v peptone; 1% w/v yeast extract; 0.5% w/v NaCl; 10% w/v PEG; 5% w/v DMSO; 10mM $MgCl_2$ and 10mM $MgSO_4$). The cells were incubated on ice for 30min prior to transformation.

3.2.6 GENERATION OF RECOMBINANT BACMIDS IN DH10BAC CELLS BY TRANSPOSITION

Between 0.1 and 0.5μg of recombinant pFASTBAC was incubated for 30min on ice with 100μl of competent DH10BAC cells. 900μl of TSBG (TSB with 20mM glucose) was added to the mixture and incubated for 4hrs with shaking to allow transposition to occur. Transformed cells were plated onto LB-agar plates containing 50μg/ml kanamycin sulphate (Roche), 12.5μg/ml tetracycline (Roche) and 7μg/ml gentamycin (Roche). Recombinants were detected by the addition of X-gal and IPTG as previously described (2.2.14). Recombinants were selected on the basis of their white colour and large colonies as described in the manufacturers protocols. Putative recombinants were replica plated (to ensure true white colonies had been selected) and were used to inoculate overnight cultures in LB-broth with the same antibiotics.

3.2.7 ISOLATION OF COMPOSITE BACMID DNA

Composite bacmid DNA was isolated according to the method described in the manufacturers protocols (Amemiya *et al*., 1994). This method is a modification of the alkaline lysis method (Birnboim and Doly, 1979). Briefly, the method involved the collection of cells and resuspension in 300μl of solution I (50mM glucose; 10mM EDTA and 25mM Tris pH 8). Cells were lysed by the addition of 300μl of lysis buffer (0.2N NaOH and 1% SDS). Protein and genomic *E. coli* DNA were removed by the addition of 300μl of 2.5M KAc pH 5.2 followed by centrifugation in a microcentrifuge. Composite bacmid DNA was precipitated out of the supernatant by the addition of 0.7 volumes of 100% isopropanol. The pellet was washed with 70% ethanol and air-dried in a laminar flow cabinet under sterile conditions. The composite bacmid DNA was resuspended in sterile UHQ.

3.2.8 TRANSFECTION INTO *SPODOPTERA FRUGIPERDA* CELLS

Spodoptera frugiperda (Sf9) tissue culture methods were performed according to O'Reilly *et al.* (1992). Sf9 cells are derived from pupal ovarian tissue of the fall armyworm and were supplied by the American Type Culture Collection (ATCC).

Sf9 cells were seeded at 9 x 10^5 in a 6-well, 35mm sterile tissue culture plate in 2ml Graces insect medium supplemented with 10% foetal calf serum (FCS); Pluronic F-68 (Palomares *et al*., 2000) and antibiotics (120μg/ml Penicillin G, 120μg/ml Streptomycin sulphate and 0.3μg/ml Fungizone). After seeding for 1hr, cells were washed with 2 x 2ml Graces medium without FCS,

Pluronic F-68 or antibiotics. 6μl of bacmid miniprep DNA was diluted in 100μl of Graces medium without FCS, Pluronic F-68 or antibiotics. This was added to 6μl of CELLFECTIN, a cationic lipid reagent optimized for insect cell transfection, diluted in Graces medium without FCS or antibiotics. Washed cells were overlaid with the bacmid / cellfection solution and incubated at 27°C for 5hrs. The transfection mixtures were removed and replaced with Graces medium containing FCS and antibiotics and allowed to incubate for 96hrs at 27°C. The medium containing the virus was collected and stored at 4°C. Transfection cells were isolated and screened for recombinant bacmid protein expression.

3.2.9 INFECTION OF SF9 CELLS

6-well, 35mm tissue culture dishes were seeded at 1.2 x 10^6 cells/well and allowed to seed for 30-60min at RT. 200μl of transfection supernatant in a 500μl volume of Graces insect medium was used to infect the cells. Following 1hr incubation at 27°C in a humid chamber, the volume was increased to 2ml with Graces medium. Following 96hrs incubation, cells were removed by pipetting and collected by centrifugation at 4000rpm in a microcentrifuge. After washing with 1ml of 1 x PBS, the cells were resuspended in 40μl of 1 x PBS. 5μl samples were diluted with 2 x PSB and denatured at 95°C for 3min. Protein expression was analyzed by SDS-PAGE.

3.2.10 VIRUS TITRATION BY PLAQUE ASSAY

The titres of transfection supernatants which expressed recombinant VP6 proteins were determined by plaque assay. 6-well, 35mm tissue culture plates were seeded at 1.8 x 10^6 cells per well. A dilution series of the transfection supernatant was prepared by diluting 10^{-1} to 10^{-9}. Medium was removed from the seeded wells and replaced with 10^{-4} to 10^{-9} dilutions. Plates were incubated at RT for 1hr. The virus dilutions were removed and replaced with 2ml of a 50% 3% low melting agarose (Promega) : 50% Graces medium mixture. Plates were incubated for 96hrs at 27°C in a humid chamber. 1ml of a 100μg/ml neutral red solution in Graces medium was added to each well and incubated for 5hrs at 27°C in a humid chamber. The neutral red solution was removed and the plates were incubated overnight. Titres were determined by counting the plaques.

3.2.11 INFECTION OF MONOLAYERS FOR VIRUS STOCKS AND PROTEIN

75cm^2 or 25cm^2 tissue culture flasks were seeded with 1 x 10^7 or 3 x 10^6 cells respectively. For virus stocks, cells were infected at 0.1pfu/cell and for protein 10pfu/cell. After incubation at 27°C for 96hrs, virus was collected from the supernatant by centrifugation at 2000rpm for 10min. Titres were determined as above.

For protein harvesting, cells were dislodged from the flask by knocking against the heel of the hand. Cells were collected by centrifugation at 4000rpm followed by washing in 1 x PBS and resuspension.

3.2.12 WESTERN IMMUNOBLOT

Western blotting described by Towbin *et al*. (1979) entails the use of antibodies which react specifically to antigenic epitopes displayed on a target protein attached to a solid support. Guinea pig anti-AHSV-3 antibodies were obtained from the Onderstepoort Veterinary Institute (OVI). Proteins were separated by SDS-PAGE and transferred to a Hybond-C extra nitrocellulose supported membrane (Amersham) using an EC-140 submerged blotter (E-C Apparatus Corporation) in Towbin transfer buffer (25mM Tris and 200mM glycine, pH 8.3). The EC-140 submerged blotter is designed for the blotting of mini-PAGE gels. However, improved

resolution of protein bands was often obtained using large PAGE gels (14 x 16cm). In certain cases, large PAGE gels were trimmed and the relevant part of the gel was blotted. Following transfer, the membrane was rinsed in 1 x PBS and non-specific binding of antibodies blocked by incubation at RT for 30min in 1% fat-free milk powder in 1 x PBS. The blocking solution was replaced with a small volume of fresh blocking solution with guinea pig α-AHSV-3 antiserum diluted 250 fold. The membrane was incubated overnight at RT with shaking in the primary antibody solution. The primary antibody was removed and the membrane was washed with three changes of washing buffer (0.05% Tween 80 in 1 x PBS). Secondary antibody, protein A conjugated horseradish peroxidase (Cappel) diluted 1000 fold, was added to the membrane and incubated with shaking for 1hr at RT. The membrane was washed three times in washing buffer followed by one 5min wash in 1 x PBS. A solution of 60mg 4-chloro-1-naphtol (Sigma) in 20ml of ice cold methanol was added to 60μl of hydrogen peroxide diluted to 100ml in 1 x PBS. The membrane was incubated in the enzyme substrate until the bands became visible.

3.2.13 5' MODIFICATION BY PCR

Life Technologies has produced two equivalent expression systems for baculovirus and bacterial protein expression. The pFASTBAC Ht vectors are a newer version of the pFASTBAC vector described above. There are 3 vectors providing the 3 possible reading frames. They include a 6-histidine tag for purification and provide a start codon. The pPROEX vectors (for bacterial expression) have the equivalent 3 vectors with identical multiple cloning sites, 6-his tag and start codon. As the start codon is provided, it is important to remove any in-phase stop codons which will result in premature termination of protein synthesis. There is an in-phase stop codon in the genome segment encoding VP6 of AHSV in the 5' non-coding region. This stop codon was removed prior to cloning into the Ht vectors. The 5' terminus of the genome segment encoding VP6 of AHSV-6 was modified by PCR as described by Nel and Huismans (1991). A primer was designed to anneal to the 5' terminal at the start codon incorporating a *Bam*HI site. The primer sequence was as follows: 5' CAGGGATCC**ATG**TCTTCGGCATTACTCC 3'. The genome segment encoding VP6 of AHSV-6 was amplified by PCR with the VP6.2 reverse primer as described previously (2.2.13). The product was digested for 3hrs with *Bam*HI, purified by the glassmilk procedure and cloned into the pGEM-3Zf(+) plasmid vector.

3.2.14 AUTOMATED SEQUENCING

pGEM-3Zf(+) plasmid (Promega) was linearized with *Bam*HI and dephosphorylated as described previously (2.2.14). The purified VP6 gene was ligated into the prepared vector and transformed into competent XL1blue cells. Recombinants were selected by blue/white selection and purified using the High pure plasmid isolation kit (Roche) according to manufacturers protocols. Half sequencing reactions were assembled as follows: 250ng of purified plasmid DNA was added to 4μl of Terminator ready reaction mix with 3.2pmoles of M13 forward or reverse primer in a final volume of 10μl. Reactions were cycle sequenced in a Perkin-Elmer GeneAmp 9600 system. The cycle was as follows: 96°C for 10sec; 50°C for 5sec and 60°C for 4min repeated for 25 cycles. Products were purified by adding 16μl UHQ and 64μl 99.9% ethanol. Tubes were vortexed and incubated for 15min at RT. Precipitated products were collected by centrifugation for 30min in a microcentrifuge followed by a 70% ethanol wash. Pellets were air-dried for 30 minutes and resuspended in loading buffer (5 parts formamide : 1 part 25mM EDTA (pH 8.0) containing 50mg/ml blue dextran). Samples were sequenced on an ABI PRISM 377 automated sequencer. Sequences were analyzed using Sequence Navigator software.

3.2.15 CLONING AND EXPRESSION IN A BACULOVIRUS SYSTEM

5' modified VP6 gene of AHSV-6 was digested out of pGEM using *Bam*HI and separated by 1% 1 x TAE agarose gel electrophoresis. The insert was excised from the agarose gel and purified by the glassmilk procedure. pFASTBAC Htb and pPROEX Htb were prepared by linearizing with *Bam*HI and dephosphorylating as described previously (2.2.14). The modified the genome segment encoding VP6 was ligated into the prepared vectors and transformed into the appropriate cells.

Following ligation into pFASTBAC Htb, the ligation mix was transformed into competent XL1blue cells as described above (2.2.7). Putative recombinants were selected on the basis of size and screened by restriction enzyme digestion with *Bam*HI and *Eco*RI. Recombinants in the correct orientation for expression were sequenced with the polyhedrin primer to verify the reading frame. Recombinant pFASTBAC DNA was transposed in competent DH10BAC cells (refer to 3.2.6). Transfection and expression proceeded as described above (3.2.8 – 3.2.11).

3.2.16 CLONING AND EXPRESSION USING A BACTERIAL SYSTEM

The pPROEX-VP6 gene ligation mixes were transformed into $CaCl_2$ competent DH5α cells. Recombinants were selected by blue/white selection on LB-agar plates containing 100μg/ml ampicillin. Possible recombinants were screened as above and recombinants in the correct orientation for expression were sequenced with the M13 reverse primer to verify the reading frame.

2ml of LB-broth containing 100μg/ml ampicillin was inoculated with a single colony containing a recombinant (or wildtype as a control) pPROEX plasmid and incubated at 37°C with shaking. 100μl of overnight culture was used to inoculate 10ml of LB-broth with 100μg/ml ampicillin. Expression was induced by the addition of IPTG to a final concentration of 0.6mM. Samples were removed at 1, 2 and 3hr intervals and optical density readings were taken at 590nm. Approximately 0.2 A_{590} (usually 2μl) samples were analyzed by PAGE. Optimal protein synthesis after induction was established at 1hr. Large scale induction was achieved by scaling up the volumes of the reaction as specified in the manufacturers protocols.

3.2.17 *IN VIVO* PROTEIN LABELLING

^{35}S methionine labelled VP6 expressed in a baculovirus system was produced as described by Uitenweerde *et al*. (1995). Sf9 cells were infected with recombinant VP6 baculovirus at a m.o.i. of 10pfu/cell. At 30h.p.i., the cells were washed and starved for 1hr with Eagles medium with antibiotics, minus methionine. The medium was replaced with 1ml of fresh medium containing 30μCi/ml of L-^{35}S-methionine and labelled for 3hrs. Cells were collected by pipetting and washed twice in 1ml 1 x PBS followed by resuspension in 1 x PBS.

Bacterially expressed proteins were labelled *in vivo* with ^{35}S methionine as described by Barik and Banerjee (1991). One millilitre of LB-broth containing 100μg/ml of ampicillin was inoculated with 10μl of overnight culture and grown for 4hrs at 37°C with shaking. IPTG to a final concentration of 0.6mM and 30μCi of ^{35}S methionine were added followed by further incubation for 2hrs. Cells were collected by centrifugation at 10 000rpm for 10min. After washing in pBS, cells were resuspended in 20μl of 1 x PBS. 2μl samples were prepared for electrophoresis by the addition of 2 x PSB.

3.2.18 SUCROSE GRADIENT ANALYSIS

Sucrose gradient analysis to determine the solubility of the VP6 proteins was performed as

described by Uitenweerde *et al*. (1995). Cytoplasmic fractions were prepared from baculovirus infected cells by lysing the cells in 0.15M STE-TX with 10 strokes of a dounce. Lysed cells were passed through a 22 gauge needle and nuclei were pelleted by centrifugation at 2000rpm for 5min. Bacterially infected cells were lysed by sonification using an Ultrasonic homogenizer as described above.

Cytoplasmic fractions were layered onto 10-40% linear sucrose gradients and centrifuged for 16hr at 40 000rpm in a swing bucket SW50.1 rotor in a Beckman L-70 ultracentrifuge. Twenty drops were collected per fraction from the bottom of the gradient using a fractionator (Hoefer Scientific Instruments). 50µl samples of each fraction were analyzed by SDS-PAGE.

3.2.19 NI-NTA COLUMN PURIFICATION

Batchwise purification as described in the Life Technologies pPROEX protocol was applied to the expressed proteins. Three buffer systems were tested. 100µl of a 50% Ni-NTA (Life Technologies) resin was pre-equilibrated with 100µl of buffer A [20mM Tris-HCl pH 8.5 at 4°C; 100mM KCl, 5mM 2-mercaptoethanol, 10% glycerol and 20mM imidazole]; buffer D [50mM potassium phosphate pH 6 at 25°C; 300mM KCl; 10% glycerol; 5mM 2-mercaptoethanol] (as recommended in the manufacturers protocol) or buffer F [20mM Tris; 0.5M NaCl; 5mM imidazole; 6M Guanidine HCl pH 7.8] (Venter *et al.*, 2000). 500µl of lysed crude cell extract was added to pre-equilibrated Ni-NTA resin and incubated at 4°C for 20min with shaking. The suspension was centrifuged at 1000rpm for 1min and the supernatant (containing material which does not bind to the resin) was removed. The resin was washed twice with 1ml of buffer A, D or F for 5min at 4°C. The protein was eluted by 5min mixes with 3 x 200µl of buffer C [20mM Tris-HCl pH 8.5 at 4°C; 100mM KCl, 5mM 2-mercaptoethanol, 10% glycerol and 100mM imidazole]; buffer E [50mM potassium phosphate pH 6 at 25°C; 300mM KCl; 10% glycerol; 5mM 2-mercaptoethanol; 100mM imidazole] or buffer G [20mM Tris; 0.5M NaCl; 0.3M imidazole pH 7.8]. The 600µl elutant was precipitated by TCA (trichloroacetic acid) or acetone precipitation.

3.2.20 GLYCOSYLATION ASSAY BY PAS STAINING

Carbohydrate-specific periodic acid Schiff (PAS) staining is a staining method developed for identification and staining of glycoproteins. PAS staining was performed as described by Carlsson (1993). Proteins were separated by SDS-PAGE. The gels were incubated for 2hrs in fixation solution (10 acetic acid: 35 methanol: 55 dH_2O) followed by 1hr incubation in periodate solution (0.7% w/v periodic acid in 5% v/v acetic acid) with shaking. The periodate solution was decanted and the gels were rinsed briefly in dH_2O. 0.2% w/v sodium meta-bisulfite in 5% v/v acetic acid was added and incubated for 5–10 min until the gels turned yellow. The solution was replaced with fresh meta-bisulfite solution and incubated for a further period of 5-10min until the gels had just decolourized. The gels were incubated in Schiff's reagent between 30min and 2hrs until red bands appeared. The staining reactions were found to be temperature sensitive and all incubations were performed at 30°C. Two positive controls were included namely the 45kDa band of Rainbow marker is ovalbumin which is glycosylated as well as glycosylated blood serum proteins.

3.3 RESULTS

In order to characterize the VP6 protein of AHSV, a number of experiments were carried out. Various expression systems were assessed including *in vitro* translation in a rabbit reticulolysate system; *in vivo* expression in a baculovirus system and bacterial expression. The proteins were authenticated by immunological screening and the solubility investigated by sucrose gradient centrifugation. Several methods of protein purification were investigated. Glycosylation was assayed using a carbohydrate specific staining assay.

3.3.1 *IN VITRO* TRANSLATION OF VP6 mRNA

To confirm that the VP6 mRNA encoded the expected VP6 proteins, the mRNA transcription product of the genome segment encoding VP6 was translated *in vitro* in a rabbit reticulolysate system. AHSV-3 and AHSV-6 VP6 genes were both cloned into the *Bam*HI site of the pBS bluescribe expression vector under control of the T3 and T7 promoters respectively (described previously in 2.2.14 and 2.3.2). Both recombinants were linearized downstream of the inserted gene using sites in the pBS multiple cloning site. Following digestion, the linearized vectors were purified by means of phenol / chloroform purification and transcribed using T3 and T7 polymerase respectively. The transcription products were resolved on a 1% 1 x TAE agarose gel (figure 3.1).

Approximately 1μg of mRNA was used for *in vitro* translation in a rabbit reticulolysate system in the presence of ^{35}S methionine. The translation products were separated on a 12% polyacrylamide gel. The dried gel was autoradiographed (figure 3.2). AHSV-3 VP6 electrophoresed at approximately 42kDa and AHSV-6 VP6 electrophoresed at approximately 43kDa. There was a notable difference in the level of expression with significantly higher levels of AHSV-6 VP6 protein expressed. For further analysis it was decided to express the proteins in a baculovirus system.

3.3.2 *IN VIVO* BACULOVIRUS EXPRESSION

For functional assays, large amounts of protein were required. The first expression system utilized was a baculovirus system using the first generation pFASTBAC vector. The genome segments encoding VP6 were cloned into the *Bam*HI site of the pFASTBAC vector. Orientation and sequence were confirmed by restriction enzyme analysis and sequencing. The recombinant vectors were used for site-specific transposition to produce recombinant baculovirus DNA (bacmids) in *E. coli* cells. Following isolation of composite bacmids, Sf9 cells were transfected using the cationic lipid reagent CELLFECTIN. 96hrs post transfection, the transfection supernatant was harvested and the virus titre was determined by plaque assays. The supernatant was used for the production of virus stocks which were used for the expression of VP6 of AHSV in Sf9 cells. Expression was analyzed on a 12% separating PAGE gel stained with Coomassie brilliant blue (figure 3.3a). AHSV-6 VP6 was expressed at a higher level

than that of AHSV-3 VP6. The difference in mobility found in the *in vitro* expression was also observed in the *in vivo* expression of VP6. In order to establish whether the protein products produced were authentic AHSV proteins, Western immunoblot analysis was performed on the expression products.

VP6 of both serotypes reacted positively with α-AHSV-3 guinea pig antiserum (figure 3.3b). Since VP6 of AHSV-3 and AHSV-6 is highly conserved, further investigations were focused on only one of these two, namely VP6 of AHSV-6.

A new generation of BAC-to-BAC vectors namely the Ht vectors were also explored for expressing VP6. VP6 expression using the pFASTBAC vector in a baculovirus system gave good levels of expression. However, ideally purified proteins were required. The new generation Life Technologies Ht vectors allow cloning into the three different reading frames (Hta – Htc) and offer a possible purification method utilizing a six histidine tag and nickel column chromatography. These vectors provide a start codon followed by a histidine tag and an rTEV protease cleavage site upstream of the multiple cloning site. The correct reading frame for the expression of VP6 was provided by Htb. In order to provide a non-disrupted ORF, the stop codon in the 5' non-coding region of the genome segment encoding AHSV-6 VP6 was removed.

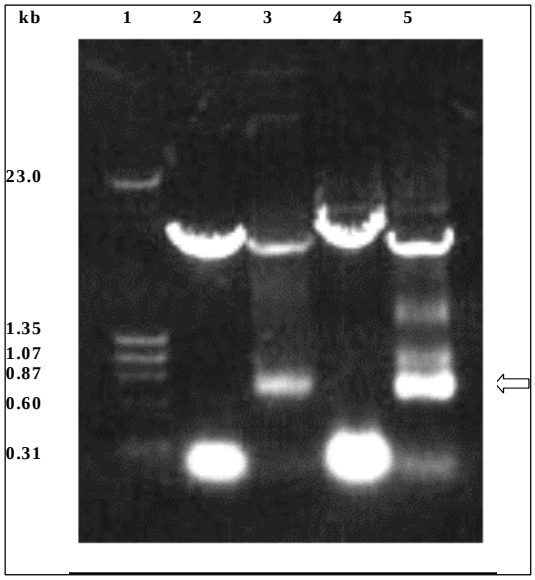

Figure 3.1 ***In vitro*** **transcription of the AHSV-3 VP6 gene (T3) and the AHSV-6 VP6 gene (T7).**

Transcription products were analyzed by 1% TAE agarose gel electrophoresis.

Lane 1: molecular weight markers;

lane 2: *Sma*I linearized AHSV-3 VP6 recombinant pBS;

lane 3: *in vitro* transcription of the AHSV-3 VP6 gene from the T3 promoter of pBS;

lane 4: *Hind*III linearized AHSV-6 VP6 recombinant pBS and

lane 5: *in vitro* transcription of the AHSV-6 VP6 gene from the T7 promoter of pBS.

The arrow indicates the mRNA of interest.

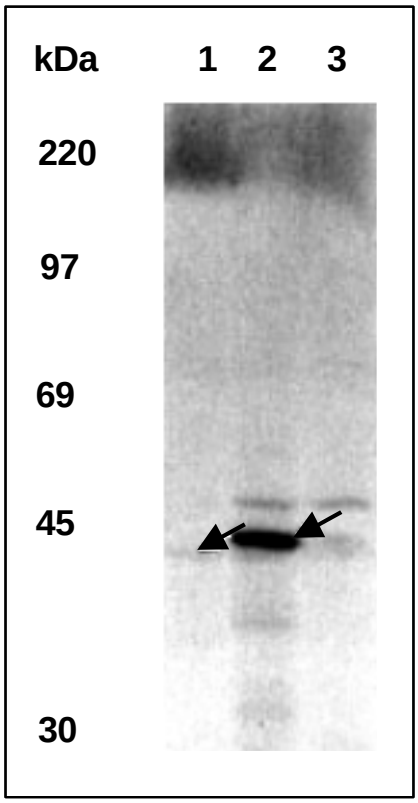

Figure 3.2 ***In vitro*** **translation of the transcription product of the genome segments encoding VP6 of AHSV-3 and AHSV-6.**

Denatured mRNA was translated in the presence of ^{35}S methionine, separated SDS-PAGE gel electrophoresis followed by autoradiography.

Rainbow marker sizes are indicated on the left in kDa.

Lane 1: rabbit reticulolysate translation of AHSV-3 VP6;

lane 2: rabbit reticulolysate translation of AHSV-6 VP6;

lane 3: a negative control with no mRNA.

An in-phase stop codon is located from nucleotides 3 – 5 in the 5' non-coding region of the genome segment encoding VP6 of AHSV. In order to remove this stop codon, a primer was designed which incorporates a *Bam*HI site and anneals from the start codon thereby removing the 5' non-coding region and stop codon. Subsequent to PCR amplification, the product was cloned into the sequencing vector pGEM-3Zf(+). The entire gene was sequenced by automated sequencing using M13 forward (T7 terminal) and reverse (SP6 terminal) primers to establish that the modification had been introduced correctly at the 5' end and that no mutations had been introduced by the PCR process.

The AHSV-6 VP6 gene with the 5' modification was digested out of pGEM and cloned into the *Bam*HI site of pFASTBAC Htb and pPROEX Htb. Insertion and orientation were determined by *Bam*HI and *Eco*RI digestions respectively. The 5' end was sequenced using the polyhedrin primer for pFASTBAC to ensure the correct reading frame. A recombinant bacmid was generated in DH10BAC cells and used for expression in Sf9 cells. Expression products were analyzed by 15% separating PAGE. The gels were stained using Coomassie brilliant blue (figure 3.4a).

3.3.3 BACTERIAL EXPRESSION

Life Technologies have a range of bacterial expression vectors (pPROEX) which are equivalent to the BAC-to-BAC Ht vectors in that they have identical multiple cloning sites for the three different reading frames i.e Hta pFASTBAC has the same multiple cloning site and reading frame as Hta pPROEX and produces the same non-viral extensions in the expressed protein. The pPROEX vectors also have a histidine tag for purification purposes. It was hoped to over-express VP6 in bacterial cells and purify the protein for further analysis. To this end, it was decided to use the pPROEX bacterial system. The AHSV-6 VP6 gene was cloned into the *Bam*HI site of the pPROEX Htb vector and insertion and orientation determined by digestions with *Bam*HI and *Eco*RI. The correct reading frame was confirmed by sequencing the 5' end using the M13 reverse primer.

pPROEX-VP6 was expressed in DH5α bacterial cells after induction with IPTG. Expression products were analyzed by 15% separating PAGE. The gels were stained using Coomassie brilliant blue (figure 3.4). The proteins appeared to be approximately the same size. Expression levels in the bacterial system appeared to be higher, however, adequate levels of expression were obtained using the baculovirus system.

Several signals for post translational modifications were found in the amino acid sequence of AHSV VP6. Should any post translational modifications occur, they may result in a modified mobility as evidenced on a SDS-PAGE gel. To this end the baculovirus and bacterially expressed proteins were separated on a 15% separating PAGE gel and stained with Coomassie brilliant blue The proteins appeared to be the same size even when loaded in the same well (results not shown).

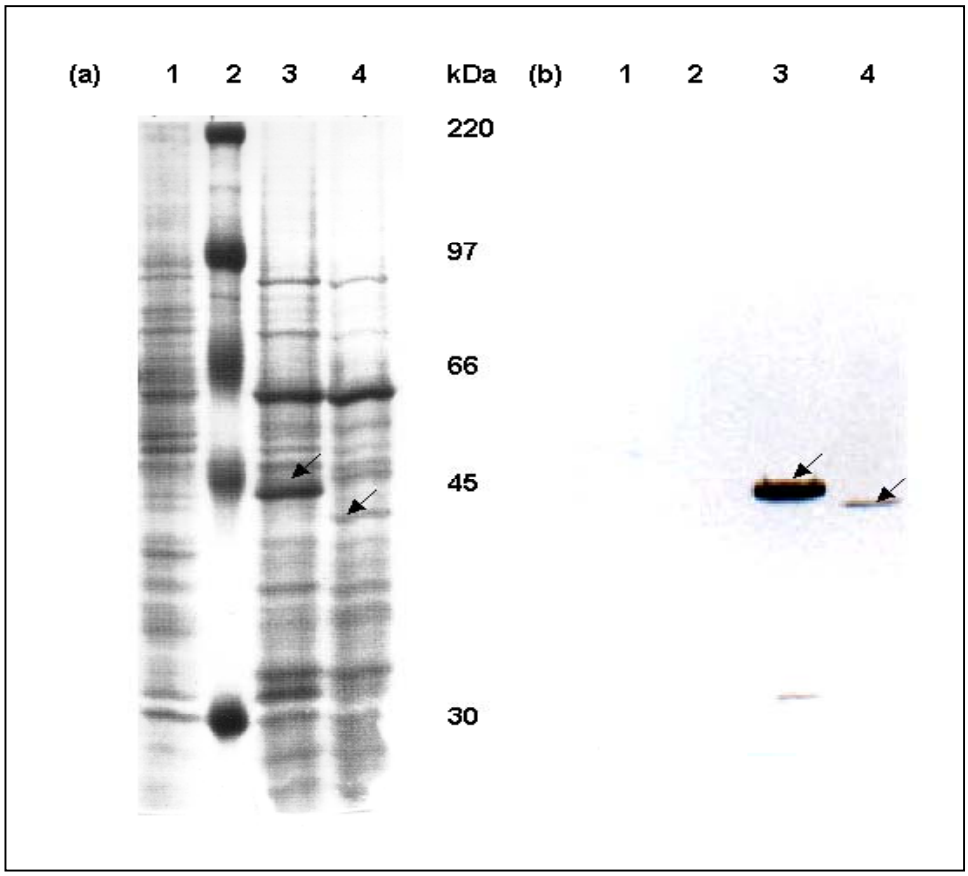

Figure 3.3 ***In vivo*** **expression and western immunoblotting of VP6 of AHSV-3 and AHSV-6.**

Proteins were expressed in Sf9 cells by means of a baculovirus VP6 recombinant and analyzed by Coomassie stained SDS-PAGE (a) and Western blot (b).

In a) lane 1: Sf cells infected with WT pFASTBAC;

lane 2: Rainbow marker;

lane 3 and 4: Sf9 cells infected with AHSV-6 and AHSV-3 recombinant VP6 respectively.

In b) lane 1: mock infected Sf9 cells,

lane 2: wildtype pFASTBAC infected Sf9 cells;

lane 3: AHSV-6 VP6 recombinant infected Sf9 cells and

lane 4: AHSV-3 VP6 recombinant infected Sf9 cells.

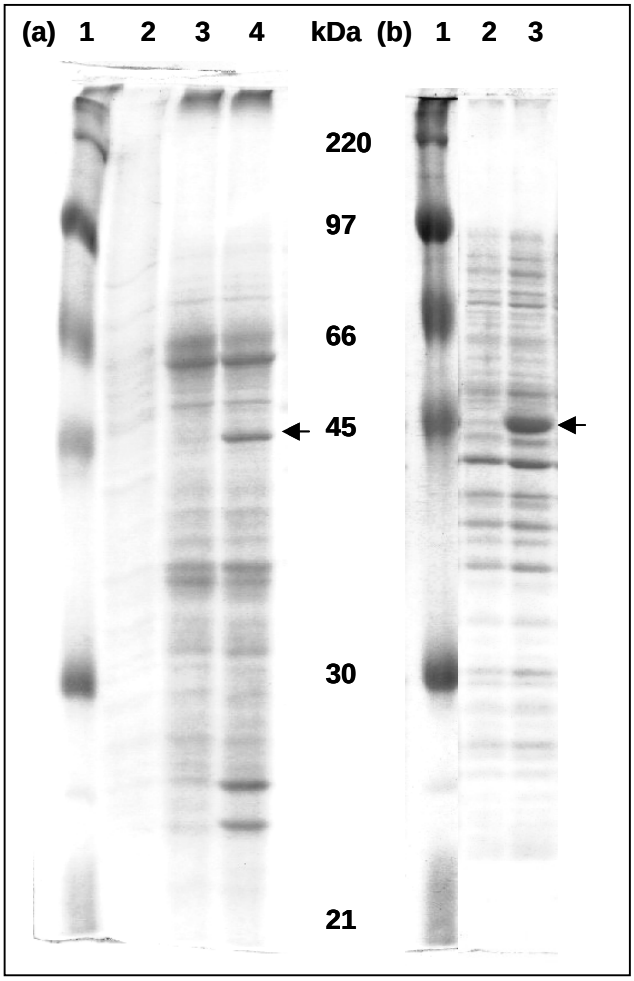

Figure 3.4 Comparison of expression of AHSV-6 VP6 in insect and bacterial cells.

VP6 was cloned into the new generation Life Technologies vectors and analyzed by Coomassie brilliant blue stained SDS-PAGE analysis. a) Baculovirus expressed VP6 and b) bacterially expressed VP6.

In a) and b) lane 1: Rainbow marker.

In a) lane 2: mock infected Sf9 cells;

lane 3: wildtype vector infected Sf9 cells;

lane 4: recombinant VP6 baculovirus infected Sf9 cells.

In b) lane 2: control bacterial cells and

lane 3: bacterially expressed VP6.

The arrow indicates the VP6 protein.

3.3.4 WESTERN IMMUNOBLOT ANALYSIS

The proteins expressed using pFASTBAC Htb and pPROEX were screened immunologically to establish authenticity. A western immunoblot was performed on baculovirus and bacterially expressed proteins (figure 3.5). The baculovirus expressed protein reacted positively to the α-AHSV-3 guinea pig antiserum, however, no reaction was obtained for bacterially expressed VP6 although some background is evident. The levels of expression of the baculovirus and bacterially expressed protein used for the immunological screening in figure 3.5 vary considerably. The baculovirus expressed VP6 shows as a very faint band on the SDS-PAGE gel compared to the clear band representing bacterially expressed VP6 (figure 3.5b). This gives a good indication of the sensitivity of the Western immunoblot (figure 3.5a). The Western blot of the bacterially expressed VP6 protein was repeated numerous times. Following the blotting of the proteins to membranes the acrylamide gels were stained with Coomassie brilliant blue to determine whether the proteins had transferred to the membrane. To all appearances, most of the protein had been transferred from the gel. However, transferred VP6 protein did not react with the antiserum.

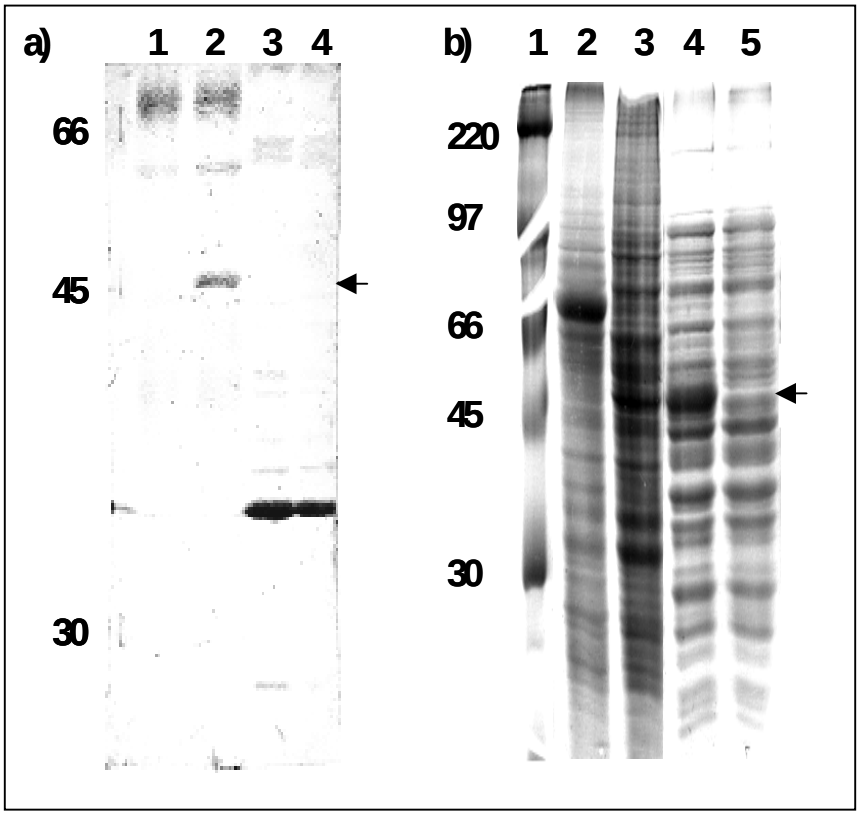

Figure 3.5 Immunological screening of baculovirus and bacterial expressed VP6.

In a) Western immunoblot using α-AHSV-3 guinea pig antiserum.

lane 1: wildtype baculovirus infected Sf9 cells,

lane 2: baculovirus expressed VP6,

lane 3: bacterially expressed VP6,

lane 4: control bacterial cells.

In b) Coomassie brilliant blue stained control SDS-PAGE gel.

lane 1: Rainbow marker

lane 2: wildtype baculovirus infected Sf9 cells,

lane 3: baculovirus expressed VP6,

lane 4: bacterially expressed VP6,

lane 5: control bacterial cells.

3.3.5 PROTEIN SOLUBILITY STUDIES

Although VP6 is expressed in relatively large amounts in bacterial cells, it needed to be considered if the protein is soluble. Baculovirus and bacterially expressed VP6 were analysed for solubility by sucrose gradient sedimentation analysis. Samples were loaded onto five step 10 – 40% linear sucrose gradients and centrifuged for sixteen hours at 40 000rpm. Sixteen fractions were collected from each gradient and samples of

fractions 5 – 16 were analyzed by PAGE. AHSV VP6 expressed in Sf9 cells was is soluble and was identified in fractions 11 – 14 at the top of the gradient (where fraction 1 was collected from the bottom of the gradient). The was no baculovirus expressed VP6 found in the pellet. This result suggests a single oligomeric structure as the VP6 protein is found in a discrete number of fractions. VP6 expressed in *E. coli* cells appears to have a higher sedimentation value as the protein is located further into the gradient with most of the protein in fractions 7 – 11 (figure 3.6). This may indicate a different oligomeric structure, for example tetramers or hexamers as found in BTV VP6 (Kar and Roy, 2003). There is a small amount of bacterially expressed VP6 in all the fractions suggesting the possibility of a range of oligomeric structures which may or may not be stable. There is some insoluble bacterially expressed VP6 in the pellet, however, most of the protein remained soluble.

3.3.6 PROTEIN PURIFICATION

Ni^{2+}-NTA (nitro-tri-acetic acid) resin protein purification is based on principles of immobilized metal chelate affinity chromatography. Various buffer systems have been developed for purification under native or denaturing conditions. A Tris-buffer system at pH 8.5 for basic proteins and a phosphate buffered system (pH 6.0) for acidic proteins were used. Buffers with 6M guanidine HCl developed for insoluble protein purification were also applied. In all cases the histidine-tagged proteins did not bind to the column and were washed out during the washing steps (results not shown). It would seem that the proteins fold in such a way that the tag is not available for binding to the Ni^{2+} resin.

In previous nucleic acid binding studies (Roy *et al*., 1990), proteins purified partially by sucrose gradient fractionation were used. In view of the problems with the histidine tag – nickel column chromatography purification method, proteins were partially purified by sucrose gradient. Fractions containing VP6 were pooled, aliquotted and stored at -70°C. In order to minimize variation between samples, the same amount of the combined fractions was used per assay.

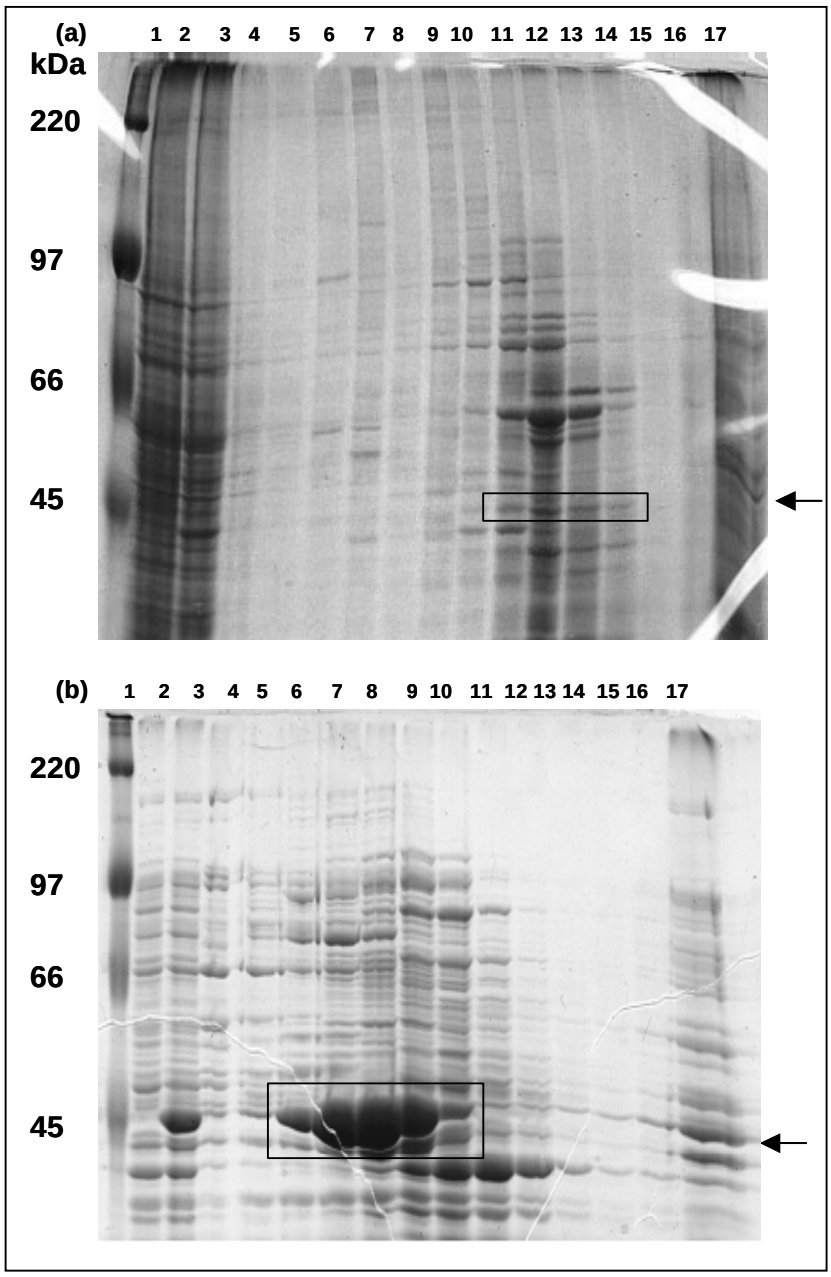

Figure 3.6 Sucrose gradient fractionation of a) baculovirus expressed VP6 and b) bacterially expressed VP6.

Samples were loaded onto 10-40% linear sucrose gradients and centrifuged for 16hrs at 40 000rpm in a Beckman SW50.1 swing bucket rotor. Fractions were separated on SDS-PAGE gels and stained with Coomassie brilliant blue.

In both a) and b)

lane 1:	Rainbow marker;
lane 2 and 3:	wildtype vector infected cell lysate and VP6 infected cell lysate respectively.
Lanes 4 – 16:	sucrose fractions 5-16 where fraction 1 was collected from the bottom of the gradient and fraction 16 from the top.
Lane 17:	a sample from the pellet.

3.3.7 GLYCOSYLATION ASSAY BY PAS STAINING

A N-glycosylation signal is located from residue 49 – 52 of the VP6 protein. In order to determine whether the expressed protein is in fact glycosylated, proteins were assayed by means of PAS staining. PAS staining is a carbohydrate specific periodic acid Schiff staining method which is an efficient method of staining glycoproteins and offers better detection of glycoproteins counterstained in Coomassie brilliant blue (Carlsson, 1993).

Baculovirus expressed VP6 was analyzed for N-glycosylation with blood serum proteins as a positive control and wildtype baculovirus as well as bacterially expressed VP6 as negative controls (figure 3.7). A positive result was obtained for baculovirus VP6 indicating that the protein expressed in a baculovirus system is glycosylated (figure 3.7a). Counterstaining with Coomassie brilliant blue (figure 3.7b) revealed that VP6 expressed in bacterial cells was present on the gel, but did not react to the PAS staining. Baculovirus VP6, although not a very distinct band on the Coomassie stained gel (figure 3.7b), is clearly evident on the PAS stained gel (figure 3.7a).

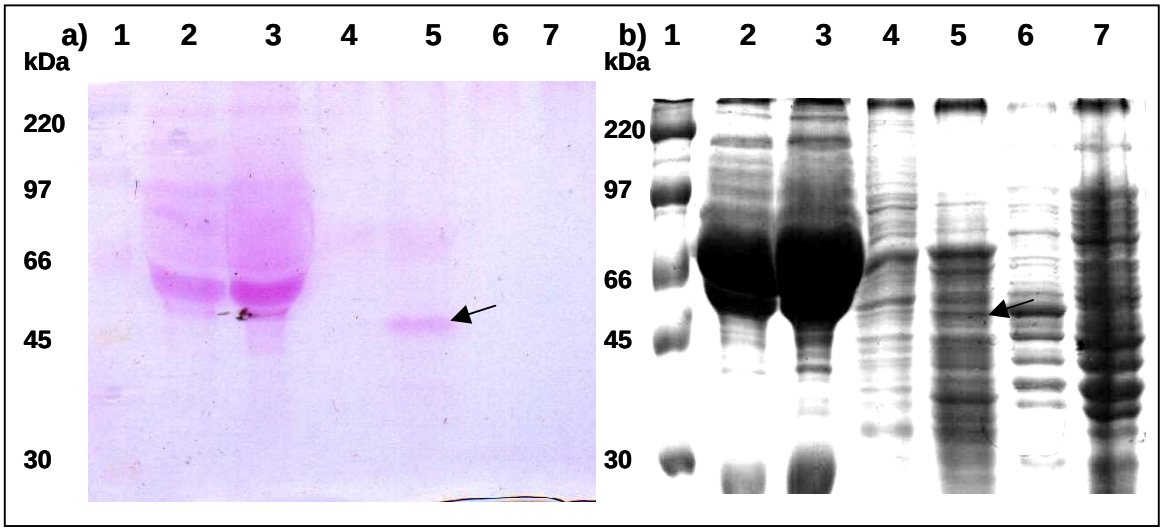

Figure 3.7 Glycosylation assay of VP6 of AHSV-6.

In a) PAS staining and b) counter staining with Coomassie brilliant blue.

Lane 1:	Rainbow marker;
lanes 2 and 3:	blood serum proteins, 5µl and 10µl respectively;
lane 4:	baculovirus wildtype infected Sf9 cells;
lane 5:	recombinant VP6 infected Sf9 cells;
lane 6:	bacterially expressed VP6 and
lane 7:	control bacterial cells.

3.4 DISCUSSION

Enzymatic functions have been assigned to the three minor inner core proteins of BTV. VP1 is the RNA polymerase; VP4 is the capping enzyme and guanylyl transferase and VP6 binds single-stranded and dsRNA and has NTPase and helicase activity. Motifs found in AHSV VP1, VP4 and VP6 suggest similar functions, however, the VP6 protein of AHSV has never been expressed or characterized with respect to such activity. In order to investigate the function of VP6, it was essential to express and characterize the protein.

The VP6 mRNA was first expressed by *in vitro* translation in a rabbit reticulolysate system. mRNA was produced by *in vitro* expression of the genome segments encoding VP6 of AHSV-3 and –6 using the pBS expression vector and T3 and T7 RNA polymerases. This mRNA was translated in a cell-free rabbit reticulolysate system in the presence of ^{35}S methionine. Unique protein bands were obtained for both serotypes. The predicted molecular weights of VP6 of serotypes 3 and 6 are 38.869kDa and 39.054kDa respectively. *In vitro* expressed AHSV-3 VP6 migrated to approximately 42kDa which is higher than the predicted M_r. VP6 of AHSV-6 migrated even higher at approximately 43kDa. This is not necessarily significant. The expressed protein corresponds well with BTV-10 VP6 that has a predicted M_r of 35.75kDa (Fukusho *et al*., 1989) but electrophoreses at 40kDa (Roy *et al*., 1990). Serotype 6 VP6 expressed at significantly higher levels than serotype 3.

The difference in electrophoretic mobility between the two serotypes as well as the difference in the levels of expression was confirmed by means of recombinant baculovirus expression. Authenticity of the proteins were confirmed by Western blot analysis using anti-AHSV-3 guinea pig antiserum. A small protein of approximately 32kDa also reacted positively in the AHSV-6 sample. This protein is most probably as a result of premature termination during translation. AHSV VP6 of serotypes 3 and 6 presents as a single band. No indication of the two forms of VP6 (VP6 and VP6a) identified in BTV-1 (Wade-Evans *et al*., 1992) was obtained.

Due to the difference in the levels of protein synthesis evidenced by *in vitro* and *in vivo* baculovirus expression and as a result of the high levels of conservation between VP6 of serotypes 3 and 6, further analysis was restricted to AHSV-6 VP6.

With the objective of obtaining large amounts of purified protein, the AHSV-6 VP6 gene was expressed in the new generation BAC-to-BAC vectors in a baculovirus system and a bacterial expression system (Life Technologies). The advantage of the pFASTBAC Ht vectors over pFASTBAC is the option to purify. For comparative purposes, pFASTBAC Htb (for baculovirus expression) is equivalent to pPROEX Htb (for bacterial expression) in terms of reading frame and the expression of non-viral protein extensions. As these vectors provide a start codon, the stop codon in the non-coding 5' end of AHSV-6 the genome segment encoding VP6 was removed by PCR. The PCR product was sequenced to ensure that no changes in sequence had been introduced by the PCR

process. Both systems generated unique bands of the expected size. VP6 was expressed at higher levels in the bacterial system than in the baculovirus system. It would appear that the levels of expression of genes under control of the baculovirus polyhedrin promoter are very much affected by the gene that is expressed. This is not necessarily due to differences in transcription levels but is more likely due to an inhibitory effect of the protein that is expressed. AHSV VP7 and NS1 were expressed at much higher levels using the same system in the same laboratory (Maree and Huismans, 1997; Maree *et al.*, 1998). VP7 is expressed at 780 molecules per virus particle compared to an estimated 60 molecules of VP6 (Mertens, 2004). The differences in protein levels in the virion may arise from signals in the sequence or alternatively reflect the relative stability in terms of denaturation and proteolysis. Sufficient VP6 was expressed in the baculovirus system to warrant continued investigation. The proteins expressed in both systems were screened immunologically to confirm their origin. The baculovirus expressed VP6 reacted positively in the Western immunoblot. However, in spite of all efforts and for no obvious reason, bacterially expressed VP6 protein could not be detected on a Western blot. This problem could not be resolved.

Hydrophilicity plots of VP6 predicted a soluble protein. The solubility of VP6 expressed in insect cells was confirmed by sucrose gradient analysis. VP6 expressed in *E. coli* cells appears to have a higher sedimentation value on sucrose gradients. The sedimentation value (S value) of a protein gives an indication of oligomerization and folding of the protein. The apparent difference in sedimentation value of baculovirus and bacterially expressed VP6 may indicate that the proteins are complexed differently. Using mass spectrophotometry, Kar and Roy (2003), found that BTV VP6 was mostly present as a monomer in solution. They found some dimers and tetramers but suggest that multimers under these conditions are unstable. By cross-linking with glutaraldehyde, they found mostly monomers and dimers but also some hexamers. VP6 of BTV appears to be capable of forming oligomers in solution but no uniform oligomeric state has been defined. In the case of AHSV VP6, it would appear that the baculovirus expressed VP6 has a single oligomeric structure under the conditions used. Following 16hrs of centrifuging at 40 000rpm, the protein localized in a discrete number of fractions. No insoluble protein was evident in the pellet. The bacterially expressed protein was recovered predominantly from fractions collected from the middle of the gradient. This suggested the possibility of a multimeric structure. This structure may be unstable which would explain the presence of some VP6 in all the fractions as this may represent the breakdown of the predominant multimer. The rapid synthesis of large amounts of proteins utilizing a bacterial system may give rise to inclusion bodies where the protein is present in mostly denatured and aggregated forms (Roy and Jones, 1996). This sedimentation value difference may be the same characteristic which affects the blotting of the bacterially expressed protein to a membrane. The majority of the bacterially expressed VP6 protein is soluble and only a small amount was identified in the pellet.

Immobilized metal chelate affinity chromatography in the form of nickel column chromatography was applied in an attempt to purify VP6 for further analysis. Three buffers namely a Tris based system with a basic pH, a phosphate based system with an acidic pH and a Tris – 6M guanidine HCl based system, developed for the purification of insoluble proteins, were investigated. In all cases, the expressed VP6 proteins failed to bind to the column. Proteins were recovered in the wash fractions. Attempts at optimizing imadazole concentrations had no effect on the result. It would seem that the 6-histidine tag was not available for binding to the column. The same results were obtained by other investigators in the same laboratory using his-tagged VP7, NS3, VP4 and NS2. VP2 was, however, purified using nickel column chromatography (Venter *et al*., 2000). Cell lysates were partially purified by sucrose gradient centrifugation.

Both phosphorylation and myristylation signals were identified in the amino acid sequence of AHSV VP6. NS2 is the only protein that has been identified as a phosphoprotein (Huismans *et al*., 1987a). In NS2 of EHDV, phosphorylation is thought to be important in down regulation of nucleic acid binding or have no effect on function (Theron *et al*., 1994). Myristoylation is usually associated with membrane bound proteins. The addition of myristate provides a mechanism for membrane binding (Towler *et al*., 1988). Such activity is likely to be of importance in membrane proteins such as NS3. It is however unlikely to play an important role in the functional activities of AHSV VP6

A glycosylation signal was found in the amino terminal of AHSV VP6. Glycosylation is found in the proteins of eukaryotes and eukaryotic viruses. An asparagine has the addition of a carbohydrate. Carbohydrates have been found to facilitate correct disulfide bond formation and protein folding (Mirazimi and Svensson, 1998). A role for glycosylation has been proposed in the assembly and secretion of hepatitis B virus middle envelope protein particles (Werr and Prange, 1998). Glycosylation has been described in P43 / hnRNP G, a RNA binding protein, which is a component of the heterogeneous ribonucleoprotein complexes. It is the first glycosylated RNA binding protein to be described (Soulard *et al*., 1993). These authors have suggested a role for glycosylation in the function or properties of the protein relating to regulation. The primary structure of P43 is characterized by a region with a high glycine content which corresponds to the primary structure of AHSV VP6. If post translational modification does occur in AHSV VP6 there may be a difference in mobility between baculovirus expressed VP6 (where post translational modification may be expected to occur) and bacterially expressed VP6 (where no post translational modification is expected). Polyacrylamide gel electrophoresis of VP6 proteins expressed in the two systems followed by staining with Coomassie brilliant blue revealed no discernible difference in mobility. According to Carlsson (1993), the relative migration of glycosylated proteins is faster in higher percentage polyacrylamide gels (such as 15%) with the result that estimated molecular weights approach the real molecular weight. Although no size difference was observed following SDS-PAGE, the difference in sedimentation value between baculovirus and bacterially expressed VP6 may be influenced by the effect of glycosylation on protein folding.

Glycosylation of baculovirus expressed VP6 was assayed using carbohydrate-specific periodic acid Schiff (PAS) staining. A clearly positive result was obtained. Characteristic of glycoproteins, the Coomassie brilliant blue stained gel showed a less distinct band than that obtained on the PAS stained gel. Coomassie brilliant blue protein staining often results in weak staining of glycoproteins (Carlsson, 1993). Periodic acid Schiff (PAS) staining is a preliminary glycosylation assay. Further, more stringent assays such as mass spectrophotometry would confirm glycosylation of AHSV VP6.

CHAPTER 4: ANALYSIS OF NUCLEIC ACID BINDING ACTIVITY OF AHSV-6 VP6

4.1 INTRODUCTION

An analysis of the AHSV VP6 sequence data indicated the presence of a helicase motif. This suggests that the protein may have helicase activity similar to BTV VP6. Mechanisms for helicase action propose an ATP-driven conformational rearrangement that generates energy. At least two nucleic acid binding sites are required in this model (Gibson and Thompson, 1994; Kadaré and Haenni, 1997). The two sites would facilitate binding to two single-stranded nucleic acid regions or one single-stranded and one duplex region at an unwinding junction. Helicases may obtain multiple nucleic acid binding sites by oligomerization (Lohman and Bjornson, 1996). In the case of dsRNA viruses, such as AHSV, it may be predicted that the putative helicase would have both dsRNA and ssRNA binding activity. The first question to be addressed was whether AHSV VP6 binds nucleic acids.

It has been reported that BTV VP6 binds viral dsRNA, ssRNA and dsDNA (Roy *et al*., 1990). These authors also localized VP6 in infected cells to the matrix of the virus inclusion bodies (VIBs), virus like particles in the VIBs as well as in the cytoplasm. In 1994, Hayama and Li demonstrated that two domains of BTV VP6, which include three of six identified antigenic epitopes, were required by BTV VP6 to bind dsRNA and dsDNA by electrophoretic mobility shift assays. In 1997, BTV VP6 was found to exhibit an RNA-dependent ATPase activity proposed to be coupled to a dsRNA helicase activity (Stäuber *et al*., 1997).

Numerous assays have been employed for the investigation of nucleic acid binding activity. The nucleic acid binding activity of BTV VP6 was investigated by means of nucleic acid overlay protein blot (north western) assays. Further questions were answered using electrophoretic mobility shift assays (EMSA). Two expression systems were utilized by the groups working on BTV VP6, namely a baculovirus system (Roy *et al*., 1990, Kar and Roy, 2003) and a bacterial system (Hayama and Li, 1994).

Results described in the previous chapter indicated that baculovirus expressed AHSV VP6 is N-glycosylated. The addition of a carbohydrate group to a protein could affect the conformation of the protein and as such affect characteristics of the protein such as the stability, solubility, protein transport, membrane interaction and the biological recognition of the protein. This raised the question of whether N-glycosylation influences the function of the protein with respect to immunological recognition and nucleic acid binding activity of AHSV VP6.

Nucleic acid binding may arise as a result of charge, conformation dependent domains or linear epitopes involved in binding. The mechanism of nucleic acid binding for AHSV

VP6 has as yet not been demonstrated. Hayama and Li (1994), used truncated deletion mutants to map and characterize antigenic epitopes and nucleic acid binding domains of BTV VP6. In order to investigate possible regions of importance for nucleic acid binding in AHSV VP6, deletion mutation analysis was performed. The conservation between two of the regions identified by Hayama and Li (1994) on BTV VP6 (70 and 71) and corresponding regions on AHSV VP6 is low. The epitope designated 75 is well conserved. Common conserved motifs found in other nucleic acid binding proteins were not identified in AHSV VP6. As a result, a series of deletion mutants were generated using restriction endonuclease sites located in the genome segment encoding VP6. The epitopes identified by Hayama and Li (1994) in BTV VP6 were accommodated within this strategy. These truncated proteins were also used to determine immunologically important regions on the AHSV VP6 protein.

The aim of the work described in this chapter was to investigate the binding activity of AHSV VP6. The effect of conditions such as salt concentration, pH and N-glycosylation on binding activity were also investigated. In an attempt to identify regions of importance for binding, a series of truncated peptides were produced and tested for binding activity.

4.2 MATERIALS AND METHODS

4.2.1 NUCLEIC ACID OVERLAY PROTEIN BLOT ASSAYS

Nucleic acid overlay protein blot assays (otherwise known as north/south western binding assays) were performed according to a combination of the methods described by Roy *et al*. (1990) and Mears and Rice (1996). Initially, PAGE gel purified proteins were used for binding assays, later partially purified proteins (by sucrose gradient fractionation) were separated by 15% PAGE. Samples containing an estimated 1μg of the desired protein and an equivalent amount of wildtype proteins were used in each assay. For each assay, samples were electrophoresed in repeats of five. The proteins were transferred to a nitrocellulose membrane using an EC140 mini submerged blotter as described previously (3.2.12). The membrane was cut into 5 identical strips containing a marker, a control and a VP6 sample. Each membrane was placed in a bag and transferred proteins were allowed to renature at 4°C overnight in standard binding buffer (SBB) (Mears and Rice, 1996). Basic standard binding buffer [50mM NaCl; 1mM EDTA; 10mM Tris-HCl, pH 7; 0.02% Ficoll (Sigma); 0.02% polyvinylpyrollidone (Sigma) and 0.02% BSA (Merck)] was prepared using DEPC UHQ and varied in a) NaCl concentration between 50 and 150mM and b) pH. Membranes were allowed to adjust to RT for 30min in fresh SBB. Radioactively labelled probes were applied to membranes and incubated with shaking for 1hr at RT. The SBB containing the probe was subsequently discarded and the membrane was washed three times for 10min each at RT with shaking. The membrane was air-dried and autoradiographed.

4.2.2 COMPETITION ASSAYS

Competition assays using unlabelled nucleic acids as competition agents were performed as follows. Initially, probe saturation points were determined by producing 10 identical membranes as described above (4.2.1). Several ssRNA and dsRNA probes were produced and pooled. The

specific activities of the pooled probes were determined and the probe concentration calculated. Each repeat was incubated with an increasing increment of either labelled ssRNA or dsRNA from 0.05ng to 0.5ng. Following autoradiography, the binding was quantified as described below and the saturation point for ssRNA and dsRNA was determined.

For the first competition assays, 4 x 5 identical repeat membranes were produced. For each set of 5, one membrane was probed with 0.25ng of ssRNA (control). The other four membranes were first incubated with 0.25ng, 0.5ng, 0.75ng or 1μg of either unlabelled ssRNA, dsRNA, ssDNA or dsDNA with shaking for 1hr at RT prior to the addition of the probe. Thereafter, 0.25ng of labelled ssRNA probe was added to each membrane and incubated for a further 1hr at RT with shaking. Membranes were washed with 50mM SBB followed by autoradiography and quantification. The percentage reduction in binding was calculated as a percentage of the control.

Binding was quantified in a manner similar to the method described by Lemay and Danis (1994) and Wang *et al*. (1996b) using a GS300 transmittance / reflectance scanning densitometer (Hoefer Scientific Instruments). Peaks were generated using Gelcompar software (Applied Maths, Kortrijk Belgium). Peaks were weighed and expressed as a percentage of the total window. Three repeats of each assay were quantified and for each repeat the peaks were weighed three times and averaged.

4.2.3 PREPARATION OF SINGLE AND DOUBLE-STRANDED NUCLEIC ACID PROBES

AHSV and BTV dsRNA (large segments) were kindly supplied by Dr M. van Niekerk and Prof H. Huismans respectively. Approximately 1μg of dsRNA was end labelled using T4 polynucleotide kinase (PNK) (Amersham) with 100μCi γ ^{32}P ATP (ICN). The final reaction volume of 50μl contained 500mM Tris-HCl, pH 7.6; 100mM $MgCl_2$; 100mM 2-mercaptoethanol and 6U of T4 PNK. The reaction was incubated at 37°C for 30min and terminated by G-75 Sephadex column chromatography.

ssRNA templates were produced by *in vitro* transcription of the genome segment encoding VP6 of AHSV-3 in pBS. Transcription occurred from the T3 promoter in the presence of 25μCi α^{32} CTP (Amersham) as the only source of CTP, as described in section 3.2.1.

DNA probes were generated by nick translating 1μg of AHSV VP6 gene DNA in the presence of 20μCi of α^{32}dCTP (Amersham). All the probes were purified from unincorporated nucleotides by G-75 Sephadex chromatography (Fernández and Garcia, 1996). The columns were washed with 1mM Tris pH 8.0 and the probes were washed through with 10mM Tris-HCl, 0.1% SDS. Twelve fractions of 100μl were collected and counted in the Beckman LS3801 scintillation counter. Peak fractions were pooled. Single-stranded DNA probes were produced by heating the dsDNA probes to 95°C for 5min followed by rapid cooling on ice immediately prior to use.

4.2.4 SPECIFIC ACTIVITY CALCULATIONS

The amount of label incorporated into each probe was determined by selective precipitation (Doyle, 1996). 1μl of each DNA and dsRNA labelling reaction was diluted 1:100 in 200mM EDTA, pH 8.0 and 3μl were spotted onto a 0.5cm^2 nitrocellulose membrane. SsRNA probes were diluted 1:10. 1μl was spotted onto a membrane. Each filter was air-dried and counted in the presence of 2.5ml scintillation cocktail. This determined the total cpm in the sample. 3μl of the same dilution was selectively precipitated with 0.5 volumes of 7.5M ammonium acetate

(RNA) or 1 volume of 4M ammonium acetate pH 4.5 (DNA) and 2 volumes of 96% ethanol.

Following a 30min incubation at -20°C and centrifugation for 5min at 15 000rpm, precipitated RNA was resuspended in 1 X TE and spotted onto a new membrane. The membrane was air-dried and counted with scintillation fluid in a Beckman LS3801 scintillation counter. This value represented the cpm incorporated into the sample.

The cpm incorporated value for DNA probes was obtained after incubating the precipitation mix on ice for 15min. This was followed by 2min at 37°C to redissolve the free dNTPs which precipitated in the previous step. The precipitated probe was collected by centrifugation for 15min at 15 000rpm. The pellet was washed in 0.5ml 0.67M ammonium acetate, pH 4.5 and 67% ethanol at RT with gentle shaking. The pellet was washed a second time in 90% ethanol, and resuspended in 1 X TE. The sample was spotted onto a new membrane, air-dried and counted in 2.5ml scintillation fluid.

The following formulas were used to calculate the percentage incorporation and the specific radioactivity of each product using the values obtained above.

$$\frac{\text{cpm incorporated}}{\text{total cpm}} \times 100 = \%\ \text{incorporation} \qquad (1)$$

$$\frac{\text{cpm incorporated} \times 33.3 \times \text{total volume of reaction}}{\text{mg input DNA}} = \text{specific activity (cpm/}\mu\text{g)} \qquad (2)$$

$$\%\ \frac{\text{incorporation} \times \text{total cpm added to reaction}}{\text{mg of dsRNA substrate in reaction}} = \text{specific activity (cpm/}\mu\text{g)} \qquad (3)$$

$$\%\ \text{incorporation} \times 99\text{ng} = \text{total ng ssRNA made} \qquad (4)$$

Equation (1) was used for the calculation of the percentage cpm incorporated for DNA (nick translation), dsRNA (5' end labelling) and ssRNA (*in vitro* transcription) probes. The specific activity of the dsDNA probes was calculated using equation (2) where 33.3 is derived from the use of 3µl of a 1:100 dilution. The specific activity of the dsRNA probes was calculated using equation (3). The specific activity of ssRNA probes was calculated using equation (4). The 99ng is derived from the following: if 1µl of a 1:10 dilution was precipitated, 10 x the value represents the cpm/µl. In a total reaction volume of 20µl, the total cpm would be 20 x this value. If 30µCi of labelled CTP at 400µCi/nmole were used, then $^{30}/_{400}$ = 0.075 nmoles of CTP were added to the reaction. If 100% incorporation occurred and CTP represents 25% of the nucleotides in the probe, then 4 x 0.075 = 0.3nmoles of nucleotides were incorporated and 0.3nmoles x 330ng/nmole (where 330ng/nmole = average molecular weight of a nucleotide) = 99ng of RNA synthesized (Titus, 1991).

4.2.5 N-GLYCOSIDASE F DEGLYCOSYLATION

A deglycosylation assay using N-Glycosidase F (Roche) enzyme as described by Hedges *et al*. (1999) was performed. Briefly, sucrose gradient purified baculovirus expressed VP6 samples were made up to a final volume of 100µl with endoglycosidase buffer (50mM sodium phosphate, pH 6.8; 20mM EDTA; 1% NP-40; 0.15% SDS, 1% β-mercaptoethanol and a protease inhibitor

cocktail of aprotinin; leupeptin and pepstatin A at 1μg/ml and phenylmethane sulfonyl fluoride (PMSF) at 5μg/ml). 200mU of N-Glycosidase F (Roche) were added to the mixture and incubated at 30°C for 16hrs in a shaking incubator. The results were analyzed by PAGE followed by PAS staining.

4.2.6 TUNICAMYCIN DEGLYCOSYLATION

Sf9 cells seeded at 1.8 x 10^6 in a six well tissue culture plate were infected with VP6 gene recombinant baculovirus stocks at 10 pfu/cell and treated with tunicamycin similar to the methods of Grubman and Lewis (1992) with modifications described by Theron *et al*. (1994). Briefly, 1h.p.i. the infections were treated with increasing concentrations of tunicamycin from 1μg; 2.5μg; 5μg; 7.5μg to 10μg/ml. Cells were harvested as usual and proteins were separated by PAGE followed by PAS staining. The optimal concentration of tunicamycin was determined and expression was scaled up to 75cm^2 flask infections.

4.2.7 DELETION MUTATION ANALYSIS

Using 5' modified VP6 gene of AHSV-6 cloned in the SP6 orientation as source plasmid, truncated gene fragments were generated using restriction endonuclease sites within the genome segment encoding VP6 (refer to figure 2.6). Restriction endonuclease digestions were performed according to manufacturers protocols (Roche).

Following ligation of the prepared fragments to the prepared vectors, ligation mixes were transformed into XL1blue cells and plated onto ampicillin and tetracycline containing plates. Recombinant pFASTBAC Ht molecules were selected following restriction endonuclease digestion and used for transposition in DH10BAC cells (refer to 3.2.4 – 3.2.7).

4.2.8 SCREENING OF COMPOSITE BACMID DNA BY PCR

Composite bacmid DNA was screened for inserts of the correct size by a standard PCR reaction. Primers annealing to the composite bacmid at the polyhedrin promoter [5' TTC CGG ATT ATT CAT ACC 3'] (polyhedrin primer) and the standard M13 reverse primer which anneals to the opposite side of the mini-*att*Tn7 allow amplification of a wildtype region of approximately 700bp which incorporates part of the *Lac*Z gene and MCS. If an insert were present the amplification product would be the size of the insert plus the wildtype band of 700bp.

The composite bacmids were tested in a Perkin Elmer 9600 system using the following programme: 3min at 93°C to denature the template followed by 30 cycles of 94°C for 45sec; 63°C for 45sec and 72°C for 5min. Amplification products were resolved on a 1% 1 x TAE gel and compared to a wildtype control.

The truncated and full-length control proteins were expressed in Sf9 cells as previously described (3.2.9 – 3.2.11). Sf9 cell lysates were separated by PAGE and transferred to a nitrocellulose membrane for immunological screening by means of a western blot as previously described (3.2.12).

4.2.9 ELECTROPHORETIC MOBILITY SHIFT ASSAYS (EMSA)

EMSA were performed as described by Hayama and Li (1994). Briefly, partially purified bacterially expressed full-length AHSV-6 VP6 protein samples containing increasing amounts of protein were prepared as follows: 1μg; 2μg; and 4μg. Two negative controls namely a) of the corresponding wildtype fraction containing bacterial proteins (1μl and 20μl) and b) 1μg of BSA (Roche). Each sample was incubated with 125ng of AHSV-6 dsRNA segments 1 – 3 (pool 1 purified on two sucrose gradients) in 100mM Tris-HCl; 50mM NaCl and 2.5mM $CaCl_2$, pH 7.0 at

30°C for 30min. The mobility shift of the dsRNA after incubation with increasing amounts of protein was assayed by 0.8% 1 x TAE agarose gel electrophoresis, stained with Ethidium bromide and visualized on a UV transilluminator (312nm tube, Vilber Lourmat).

4.3 RESULTS

In order to function as a helicase, AHSV VP6 would need to have nucleic acid binding activity. Such activity has been demonstrated for BTV VP6 (Roy *et al*., 1990, Hayama and Li, 1994).

In the previous chapter, the expression of AHSV VP6 in a baculovirus and a bacterial system was described. The first objective was to test for nucleic acid binding activity using AHSV VP6 expressed in both systems.

4.3.1 NUCLEIC ACID OVERLAY PROTEIN BLOT ASSAYS

Numerous groups have successfully employed nucleic acid overlay protein blot assays (north / south western blots) for the investigation of nucleic acid binding (Roy *et al*., 1990; Brantley and Hunt, 1993; Labbé *et al*., 1994; Lemay and Danis, 1994; Fernández *et al*., 1995; Bleykasten *et al*., 1996; Mears and Rice, 1996; Wang *et al*., 1996b) to name a few.

Roy *et al*. (1990), reported the use of BTV VP6 that was partially purified by sucrose gradient fractionation for RNA overlay protein blot (north western) assays. Proteins were accordingly partially purified by sucrose gradient purification, separated by PAGE and blotted to nitrocellulose membranes. Probes were generated by radioactively labelling single-stranded and double-stranded RNA and DNA.

AHSV-6 VP6 was expressed in baculovirus and bacterial systems. A preliminary binding assay with γ^{32}-P labelled AHSV dsRNA (end labelled by the addition of a γ^{32}-P ATP molecule) was performed to determine whether AHSV VP6 was able to bind to the dsRNA (figure 4.1). The results show that the baculovirus expressed VP6 was able to bind to the dsRNA with no background binding in the negative control. However, the bacterially expressed VP6 did not bind to the dsRNA in the same assay. All possible controls were in place to ensure the integrity of the bacterially expressed VP6 protein. The same 5' modified VP6 gene was used as source DNA to clone into both baculovirus and bacterial expression vectors. Restriction enzyme analysis was followed by sequencing of the 5' terminal to verify the reading frame. Unique proteins of the same size were obtained. A Western blot on bacterially expressed VP6 could not be performed successfully (refer to section 3.3.4, figure 3.5). Both Western blotting and nucleic acid overlay protein blot assays rely on transfer of proteins separated by SDS PAGE to a membrane. This could be the common underlying factor. Alternatively, there could be something different about the proteins expressed in the two different systems, for example, post- or co-translational modification. Both possibilities were investigated and will be described later. The effect of glycosylation on AHSV VP6 nucleic acid binding activity was investigated (refer to section 4.3.5) and electrophoretic mobility shift

assays (EMSA) were used to determine whether bacterially expressed proteins retained nucleic acid binding activity (refer to section 4.3.12).

The first question addressed was, however, the affinity of the baculovirus expressed VP6 for different nucleic acids.

4.3.2 AFFINITY OF AHSV VP6 FOR DIFFERENT NUCLEIC ACIDS

Roy *et al*. (1990), demonstrated that sucrose gradient purified BTV VP6 bound single-stranded and dsRNA and dsDNA. The objective was to investigate the affinity of AHSV VP6 for AHSV single and double-stranded RNA and DNA as well as a non-specific nucleic acid such as BTV dsRNA. Binding assays were performed as described in section 4.2.1 in SBB (standard binding buffer) with 50mM NaCl, pH 7.

DsRNA probes were prepared by end labelling with γ^{32}P ATP using T4 polynucleotide kinase, ssRNA probes were prepared by *in vitro* transcription of the genome segment encoding VP6 of AHSV-3 in the presence of α^{32}P CTP and DNA probes were prepared by nick translation of the genome segment encoding VP6 of AHSV in the presence of α^{32}P dCTP. SsDNA probes were prepared by denaturing nick translated dsDNA at 95°C for 5 minutes prior to application. All probes were purified from unincorporated free nucleotides by G75 Sephadex chromatography. Specific activities were determined for each type of probe and found to be in the order of dsRNA: $\pm$ 3 x 10^{10} cpm/μg; ssRNA: $\pm$ 1 x 10^{6} cpm/μg; dsDNA and ssDNA: $\pm$ 3 x 10^{6} cpm/μg.

In order to test how the different nucleic acids reacted with AHSV VP6, a nucleic acid overlay protein blot assay was performed. Five repeats of sucrose gradient purified protein were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The five repeats were separated and each membrane was equilibrated in SBB. Each membrane was probed with a different labelled nucleic acid probe. AHSV VP6 was found to bind single-stranded and dsRNA and DNA as well as BTV dsRNA in (figure 4.2). This is not a quantitative experiment and strict standardization of the amount of labelled probe and uniform specific activity of each different probe added to each membrane was not applied. Due to differences in labelling method, isotope and specific activities of the probes, the five lanes (A-E) are not comparable. What this result shows is that AHSV VP6 binds different types of nucleic acids well.

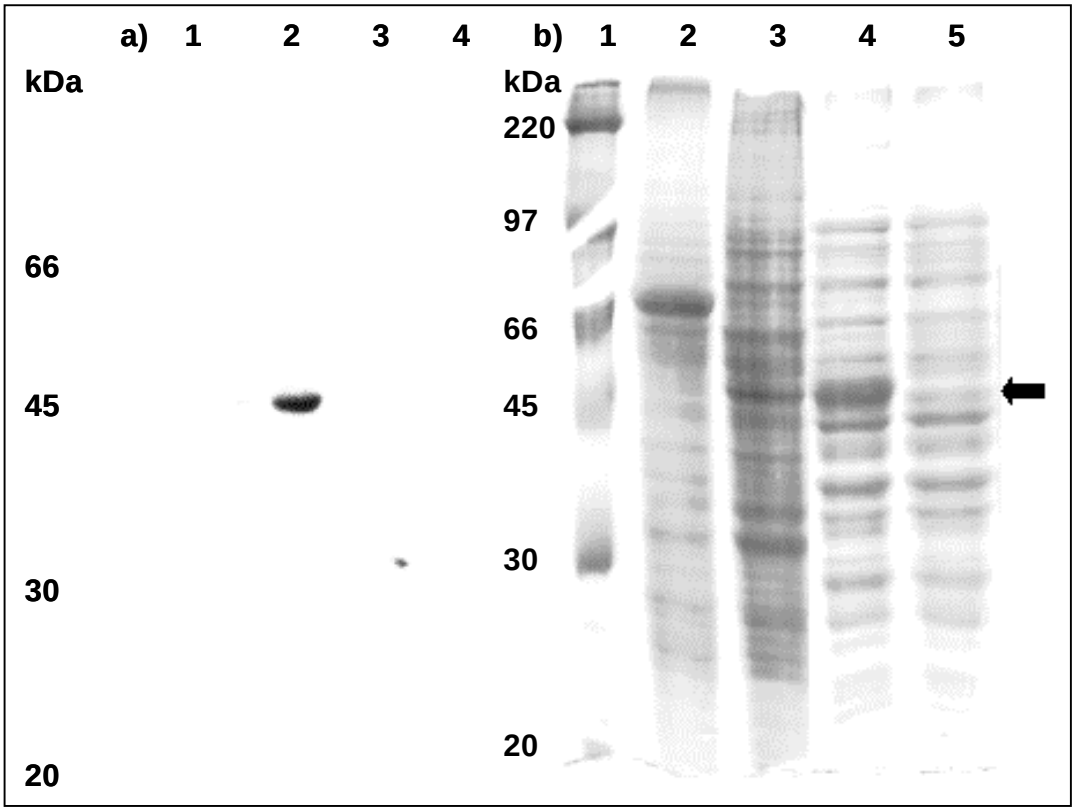

Figure 4.1 Comparison of nucleic acid binding activity of baculovirus and bacterially expressed AHSV VP6.

(a) Nucleic acid overlay protein blot assay of baculovirus and bacterially expressed VP6 probed with a dsRNA probe and (b) Coomassie brilliant blue stained SDS-PAGE control gel.

In a) Lane 1: wildtype infected Sf9 cells;

lane 2: Sf9 cells infected with a baculovirus recombinant expressing VP6;

lane 3: *E. coli* cells expressing VP6 and

lane 4: control *E.coli* cells.

In b) Lane 1: Rainbow marker;

lane 2: wildtype infected Sf9 cells;

lane 3: Sf9 cells infected with a baculovirus recombinant expressing VP6;

lane 4: *E. coli* cells expressing VP6 and

lane 5: control *E.coli* cells.

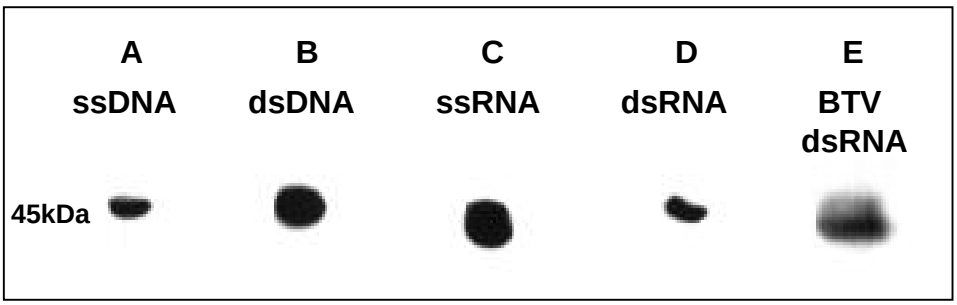

Figure 4.2 Autoradiogram of an assay to determine the affinity of AHSV-6 VP6 to various nucleic acid probes.

Sf9 cells infected with a baculovirus recombinant expressing VP6 purified by sucrose gradient fractionation were separated by SDS-PAGE and blotted onto individual membranes. Nucleic acid overlay protein blots membranes were incubated in SBB with 50mM NaCl, pH 7.

Membrane A was probed with α^{32}P labelled ssDNA;

membrane B was probed with α^{32}P labelled dsDNA;

membrane C was probed with α^{32}P labelled ssRNA,

membrane D was probed with γ^{32}P labelled AHSV dsRNA and

membrane E with γ^{32}P labelled BTV dsRNA.

4.3.3 EFFECT OF SALT CONCENTRATION ON BINDING ACTIVITY

Nucleic acid binding activity may be as a result of conformation dependent domains, linear domains or may be related to charge. Macromolecules such as nucleic acids and proteins may carry a substantial net charge depending on the pH of the solution. These molecules attract oppositely charged small ions resulting in the neutralization of net charge on the molecule (Mathews and van Holde, 1991). High salt concentrations will, therefore, affect charge and play an important role in the efficiency of charge related binding.

Our objective was to determine the effect of NaCl concentration on the nucleic acid binding activity of AHSV VP6. The binding to single-stranded and double-stranded RNA and DNA at a series of NaCl concentrations was assayed (figure 4.3a). For each type of nucleic acid, five repeats of Sf9 cells infected with a baculovirus recombinant expressing VP6 purified by sucrose gradient fractionation were separated by SDS-PAGE and blotted to generate five identical membranes. Each membrane was incubated in standard binding buffer with one of five NaCl concentrations ranging from 50mM to 150mM. Each series of membranes was probed with equal amounts of labelled probe. Following washing, drying and autoradiography, binding was quantified using a GS300 Transmittance / Reflectance Scanning densitometer (Hoefer Scientific Instruments) and Gelcompar software (Applied Maths, Kortrijk, Belgium). In figure 4.3,

a) is a sample assay and b) is the average of the quantification of three repeats of the same assay.

Maximum binding for all four nucleic acid species occurred at a concentration of 50mM NaCl (figure 4.3a and b). Therefore, decrease in binding activity was measured as a percentage of binding at 50mM. Binding decreased continuously with increased NaCl concentration. At 75mM, AHSV VP6 bound about 80% of the dsRNA, ssRNA and ssDNA that was bound at 50mM salt concentration. At 75mM, 69% of the dsDNA was bound by VP6. At 125mM, VP6 no longer bound ssDNA while dsRNA, dsDNA and ssRNA bound at ± 39%, 27% and 20% respectively of that bound at 50mM. At 150mM, dsRNA and dsDNA were bound at ±19% and 17% respectively while ±6% ssRNA was bound (figure 4.3b).

Salt concentration has a marked effect on the binding activity of AHSV VP6. Most nucleic acid overlay protein blot assays are performed at 50mM salt concentration. These results have shown that when the salt concentration in this binding assay is increased, the nucleic acid binding is strongly affected.

Having established that AHSV VP6 binds various nucleic acids and this binding is negatively influenced by increased salt concentrations, the next step was to investigate whether a preference for one type of nucleic acid over another could be established.

4.3.4 INVESTIGATION OF NUCLEIC ACID PREFERENCE

If VP6 functions as the helicase of AHSV, a preference for binding RNA over DNA may be expected. The objective was to determine whether a nucleic acid preference could be determined for AHSV VP6.

Saturation points were estimated for ssRNA and dsRNA. For both probes, ten repeats of Sf9 cells infected with a baculovirus recombinant expressing VP6 purified by sucrose gradient fractionation were separated by SDS-PAGE and blotted to produce ten identical membranes. Each membrane was incubated in standard binding buffer with 50mM NaCl, pH 7. Each membrane was probed with an increasing amount of labelled probe. Following washing, drying and autoradiography, binding was quantified. Binding saturation points for 1μg of VP6 were estimated at 0.2ng for dsRNA and 0.25ng for ssRNA.

Competition assays were performed in SBB with 50mM NaCl, pH 7 with either a ssRNA probe or a dsRNA probe. Initially, a labelled ssRNA probe was used in the presence of increasing amounts of unlabelled ssRNA, dsRNA, ssDNA and dsDNA. Unlabelled nucleic acid was added before the probe was applied to the membrane according to the saturation point established for the two probes as follows: equal amounts of unlabelled nucleic acid (1 x); twice as much unlabelled nucleic acid (2x); three times (3x) and four times (4x) as much unlabelled nucleic acid. The purpose was to investigate whether a

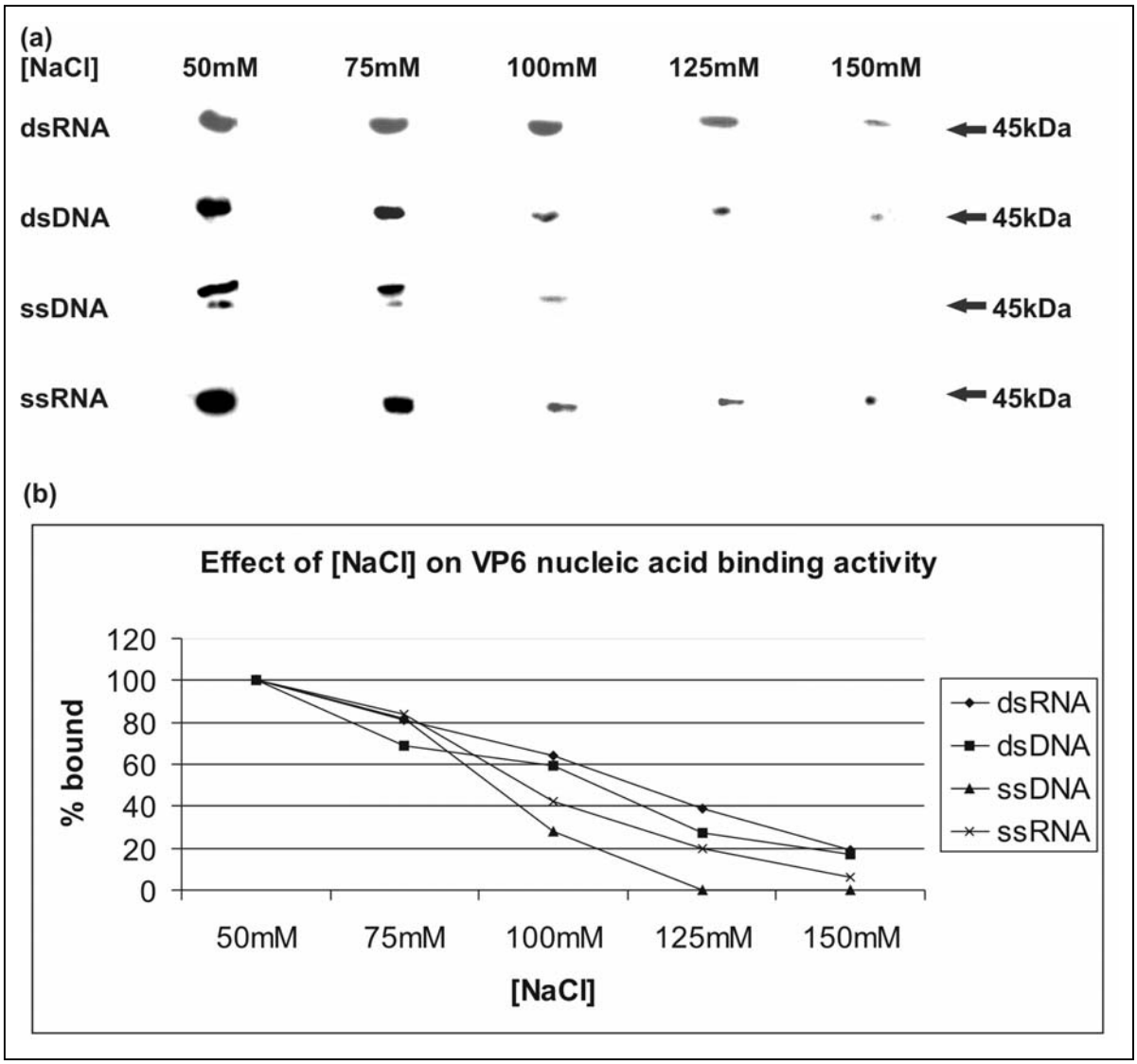

Figure 4.3 Investigation of the effect of salt concentration on VP6 nucleic acid binding activities.

(a) Autoradiograms of nucleic acid overlay protein blots using dsRNA, dsDNA, ssDNA and ssRNA probes at 5 NaCl concentrations ranging from 50mM to 150mM. Sf9 cells infected with a recombinant baculovirus expressing AHSV-6 VP6 were loaded in 5 repeats alongside markers and separated by SDS-PAGE. Proteins were blotted onto membranes and 5 strips of rainbow marker and VP6 were cut off. For each nucleic acid, an equal amount of probe was added to each membrane at a different salt concentration. Horizontal strips represent the area on the autoradiograph corresponding to the 45kDa ovalbumin of Rainbow marker with the 5 membranes aligned according to the marker. (b) Graph representing the effect of NaCl concentration on AHSV-6 VP6 nucleic acid binding quantified using a densitometer and expressed as a percentage of the binding at 50mM NaCl (average of three repeats).

preference for RNA over DNA could be established. In the second competition assay a dsRNA probe was applied in the presence of unlabelled ssRNA and unlabelled dsRNA to determine whether a preference for ssRNA or dsRNA could be established. Each assay was repeated three times. In both competition assays, reduction of binding was measured as a percentage of the control which in each assay consisted of the probe without the competition agent. Binding was quantified using a GS300 Transmittance / Reflectance Scanning densitometer (Hoefer Scientific Instruments) and Gelcompar software (Applied Maths, Kortrijk, Belgium).

A ssRNA probe was applied and unlabelled ssRNA, dsRNA, ssDNA and dsDNA were used as competition agents (figure 4.4). Both single-stranded and double-stranded DNA did not result in a large reduction in the binding of the ssRNA probe (figure 4.4). After the addition of 4x dsDNA, binding was reduced by less than 30%. The addition of 3x unlabelled DNA resulted in a reduction of less than 20% in the binding. The addition of increasing amounts of unlabelled ssRNA caused a continuous decrease in the binding of labelled ssRNA as expected. DsRNA caused an initially larger decrease in labelled ssRNA binding (1x dsRNA reduced binding by 33% as opposed to a 22% reduction caused by the addition of the same amount of unlabelled ssRNA). This decrease evened out with a 62% (dsRNA) and 63% (ssRNA) reduction in binding of labelled ssRNA after the addition of 4x competition agent. From these results it appears that VP6 has a preference for binding RNA over DNA. This is in line with what would be expected of a putative RNA helicase.

With the objective of comparing AHSV VP6 affinity for ssRNA and dsRNA, competition assays were performed with a labelled dsRNA probe and unlabelled ssRNA and dsRNA (figure 4.5). The reduction of binding was consistent for both ssRNA and dsRNA with the addition of unlabelled nucleic acid. There was once again initially a greater decrease of binding following the 1x and 2x unlabelled dsRNA than found using unlabelled ssRNA. When 4x dsRNA was added, binding was reduced by 41%, the equivalent addition of ssRNA as competition agent resulted in a 35% decrease in binding. These results suggest the possibility of a slight preference for binding dsRNA by AHSV VP6.

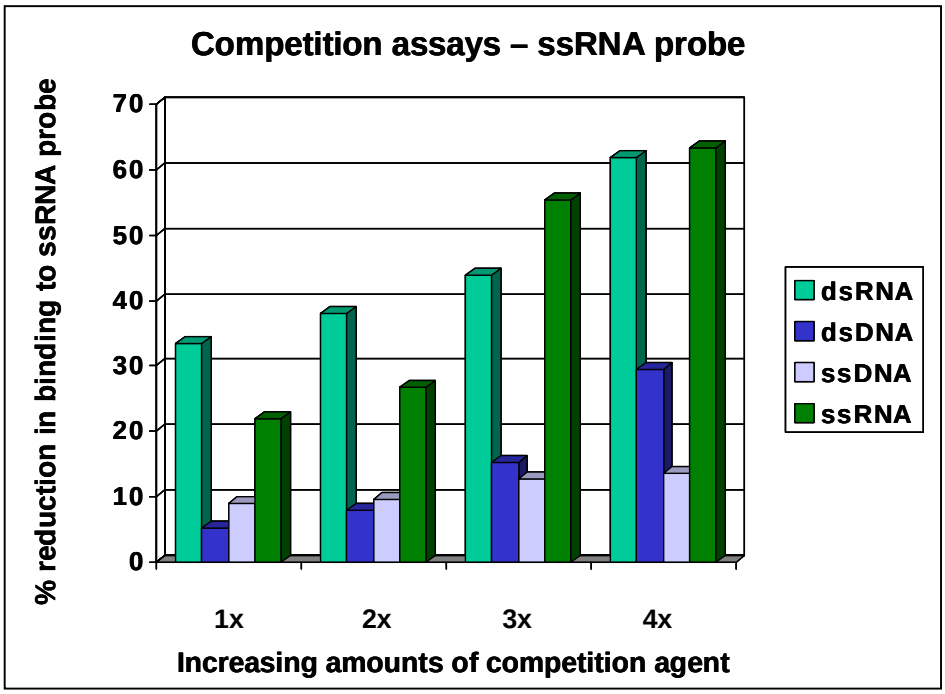

Figure 4.4 Competition assays to investigate a nucleic acid binding preference for AHSV VP6 using a ssRNA probe.

Graph indicating the percentage reduction in binding of labelled ssRNA by VP6 in the presence of increasing amounts of competition agent compared to the binding of the control (average of three repeats). Binding was assayed by nucleic acid overlay protein blots. The control membrane was probed with 0.25ng of ssRNA probe. 1x, 2x, 3x and 4x indicate the percentage reduction in binding of the probe to VP6 with prior incubation with 0.25ng, 0.5ng, 0.75ng and 1ng of unlabelled nucleic acid respectively.

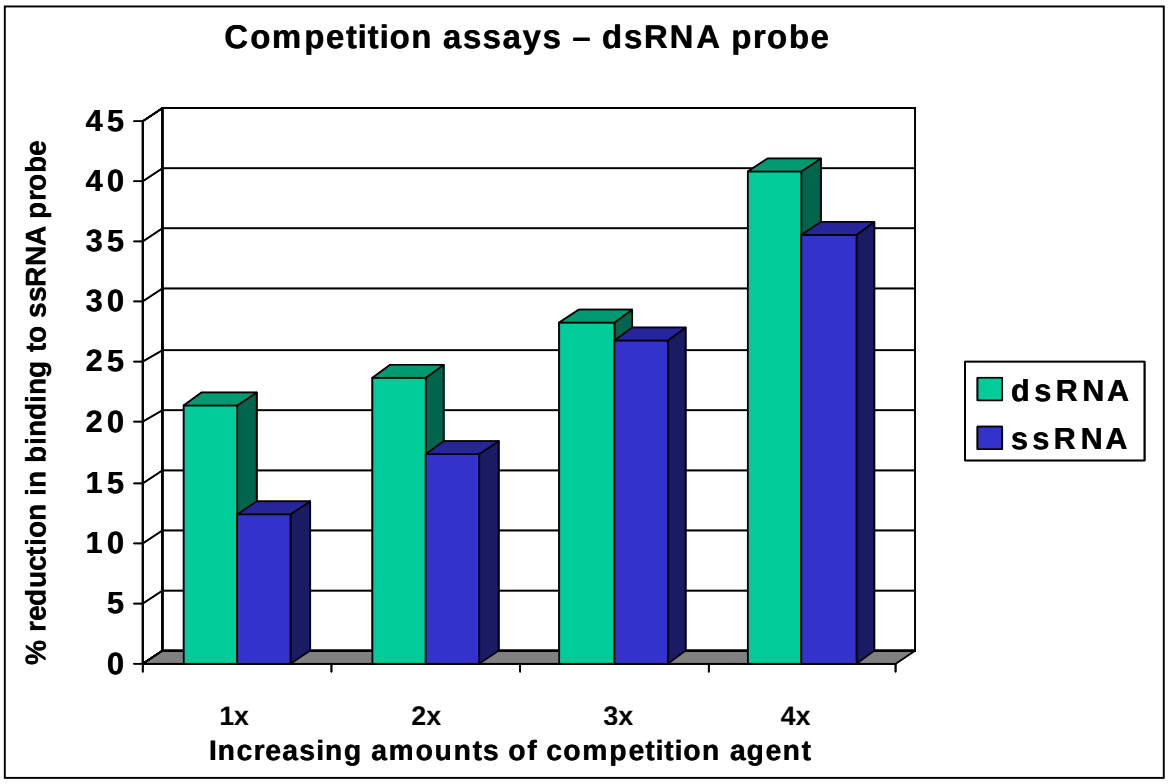

Figure 4.5 Competition studies for binding preference between ssRNA and dsRNA by AHSV VP6.

Graph indicating the percentage reduction in binding of labelled dsRNA by VP6 in the presence of increasing amounts of competition agent compared to the binding of the control (average of three repeats). Membranes were probed with 0.2ng of labelled dsRNA with increasing amounts of unlabelled ssRNA and dsRNA as competition agents {i.e. 0.2ng (1x); 0.4ng (2x); 0.6ng (3x) and 0.8ng (4x)}.

4.3.5 DEGLYCOSYLATION OF BACULOVIRUS EXPRESSED VP6

Soulard *et al*. (1993), described a glycosylated RNA binding protein P43 (hnRNP G). They suggested that the glycosylation may be important in the regulation of binding activity. AHSV VP6 expressed in a baculovirus system is glycosylated. Bacterially expressed AHSV VP6 is not glycosylated as glycosylation does not occur in prokaryotes. It was found that bacterially expressed VP6 did not bind a dsRNA probe in a nucleic acid overlay protein blot assay (refer to section 4.3.1). In view of the work done on P43 and the problems experienced with nucleic acid binding assays using bacterially expressed VP6, the question arose of whether glycosylation of AHSV VP6 plays a role in the binding activity of the protein.

In order to investigate the role of glycosylation in AHSV VP6 binding activity, the baculovirus expressed VP6 was deglycosylated and its binding activity was compared to that of the glycosylated VP6. The first step was to successfully deglycosylate baculovirus VP6. Two strategies for deglycosylating baculovirus expressed VP6 were pursued. Initially, a deglycosylation assay using N-Glycosidase F (Roche) enzyme as

described by Hedges *et al*. (1999) was performed. N-Glycosidase F cleaves asparagine bound N-glycans under certain conditions. It is, however, difficult to determine whether a protein has been entirely deglycosylated or whether it has only been deglycosylated sufficiently to avoid detection by PAS staining. Another difficulty arose as a result of the 16hr incubation at 30° C, which in spite of the protease inhibitor cocktail present, resulted in significant protein degradation.

In the light of these obstacles, tunicamycin was used to block the addition of all N-linked side chains to usually glycosylated cellular proteins (Gallagher *et al*., 1992). Tunicamycin may inhibit protein synthesis, however, all cell systems are not tunicamycin sensitive (Elbein, 1987).

To establish optimal concentrations of tunicamycin required for inhibition of the N-glycosylation pathway and to establish the effect of tunicamycin (if any) on VP6 protein synthesis in Sf9 cells, cells were inoculated with VP6 recombinant bacmid in the presence of increasing amounts of tunicamycin (1μg; 2.5μg; 5μg; 7.5μg and 10μg/ml). Coomassie brilliant blue staining revealed that there was no inhibition of protein synthesis by the addition of tunicamycin. Moreover, 2.5μg/ml was sufficient for inhibition of glycosylation of VP6 expressed in Sf9 cells as evidenced by PAS staining (results not shown).

4.3.6 EFFECT OF DEGLYCOSYLATION ON VP6 SOLUBILITY

According to Elbein (1987), N-glycosylation may increase the solubility of a protein and as such the removal of the glycosyl group may decrease the solubility. Baculovirus expressed VP6 in its glycosylated form was found to be soluble and was recovered from four fractions (11 – 14) at the top of the sucrose gradient (refer to section 3.3.5, figure 3.6a). The solubility of tunicamycin treated AHSV VP6 was also investigated by sucrose gradient fractionation (figure 4.6). The deglycosylated VP6 was also found at the top of the gradient (fractions 8 – 10, lanes 11 – 13 in figure 4.6) indicating that deglycosylation has no effect on the solubility of the protein.

4.3.7 INVESTIGATION OF THE ROLE OF N-LINKED GLYCOSYLATION OF VP6

Levels of protein synthesis may be negatively affected by Tunicamycin treatment (Elbein, 1987). Figure 4.7a shows expression of baculovirus VP6 untreated and treated with tunicamycin. It is clearly evident that the expression levels are not affected. In order to investigate a possible role for glycosylation in the function of AHSV VP6, immunological recognition and nucleic acid binding of deglycosylated baculovirus expressed VP6 were assessed. A Western immunoblot was performed to establish whether glycosylation contributed to recognition of VP6 by guinea pig anti-AHSV-3 antiserum (figure 4.7b). In the western immunoblot, tunicamycin treated VP6 reacted positively indicating that antibody recognition on a western blot is not influenced by the addition of carbohydrate to the protein. In order to assess the role of glycosylation in the nucleic acid binding activity of VP6, a nucleic acid overlay protein blot binding assay

with dsRNA as a probe was performed. Both glycosylated and unglycosylated VP6 bound the dsRNA probe with equivalent efficiency (figure 4.7c). This result indicates that glycosylation has no direct role in the binding activity of baculovirus expressed AHSV VP6. Post- or co-translational modification is, therefore, not the explanation for the lack of binding activity of bacterially expressed VP6 in a nucleic acid overlay protein blot binding assay.

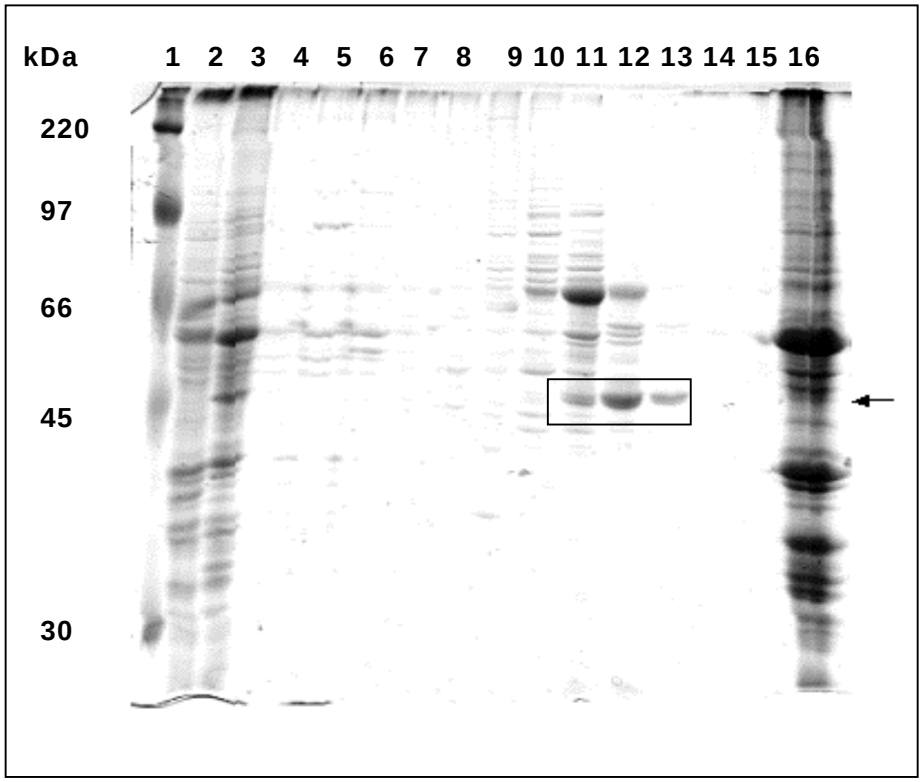

Figure 4.6 Sucrose gradient fractionation of baculovirus expressed VP6 treated with tunicamycin.

A Coomassie brilliant blue stained SDS-PAGE gel of fractions collected from a sucrose gradient where samples were loaded onto a linear 10 – 40% sucrose gradient and centrifuged for 16hrs at 40 000rpm in a SW50.1 rotor in a Beckman L-70 ultracentrifuge.

Lane 1:	Rainbow marker;
lane 2 and 3:	wildtype vector infected cell lysate and tunicamycin treated recombinant baculovirus expressing VP6 infected cell lysate respectively;
lanes 4 – 15:	sucrose fractions 1- 12 (where fraction 1 was collected from the bottom of the gradient and fraction 12 from the top);
Lane 16:	is a sample from the pellet.

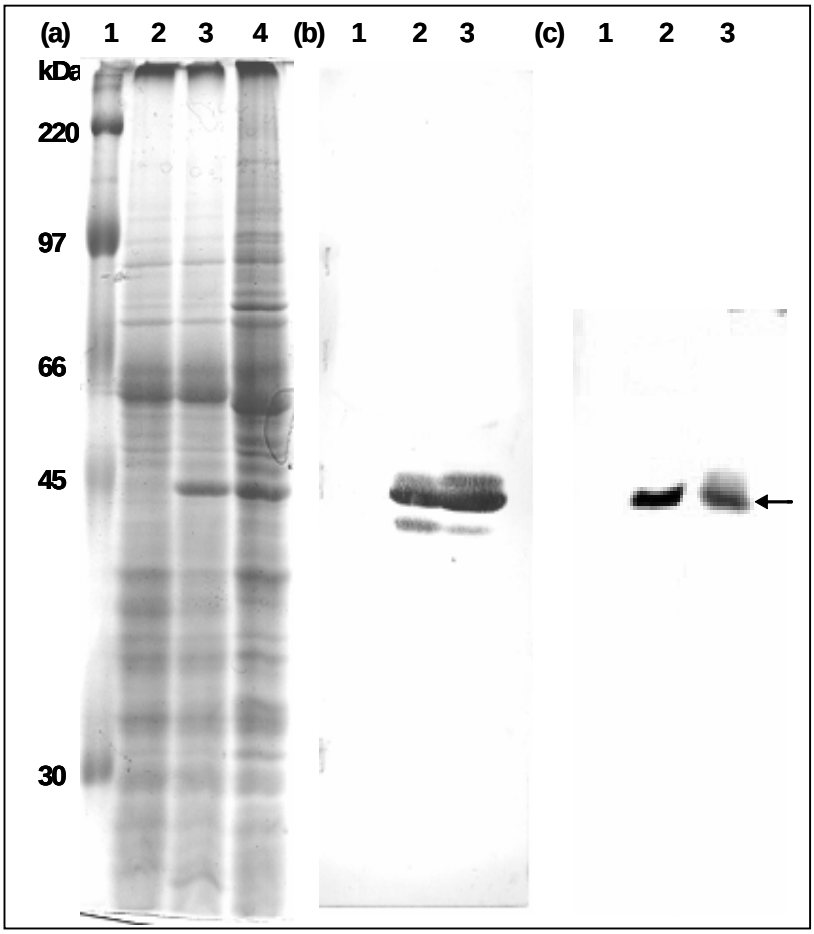

Figure 4.7 Analysis of the role of N-glycosylation in AHSV VP6 nucleic acid binding activity.

In (a), SDS-PAGE control gel stained with Coomassie brilliant blue showing protein expression.

Lane 1: Rainbow marker;

lane 2: wildtype baculovirus infected Sf9 cells;

lane 3: Sf9 cells infected with a recombinant baculovirus expressing VP6

lane 4: Sf9 cells infected with a recombinant baculovirus expressing VP6 treated with 2.5μg/ml tunicamycin.

In (b) and (c) where (b) Western immunoblot to assess immunological recognition and (c) Nucleic acid overlay protein blot assay using a dsRNA probe and proteins partially purified by sucrose gradient fractionation.

Lane 1: wildtype baculovirus infected Sf9 cells;

lane 2: Sf9 cells infected with a recombinant baculovirus expressing VP6

lane 3: Sf9 cells infected with a recombinant baculovirus expressing VP6 treated with 2.5μg/ml tunicamycin.

4.3.8 PREPARATION OF BACULOVIRUS RECOMBINANTS THAT EXPRESS DIFFERENT TRUNCATED VP6 PEPTIDES.

Truncated proteins are useful in the identification of regions involved in nucleic acid binding. In order to investigate which regions are important for nucleic acid binding, a series of VP6 gene deletions were cloned and the truncated proteins expressed in Sf9 cells.

Analysis of the primary structure of AHSV VP6 did not reveal common motifs found in other nucleic acid binding proteins. Two of the domains involved in binding identified by Hayama and Li (1994) in BTV VP6, namely 70 and 71, were not well conserved in AHSV VP6. The third domain designated 75, is well conserved in AHSV, BTV, Chuzan and St Croix River virus VP6. A series of deletion mutants were generated using restriction enzyme sites in the gene (refer to figure 2.6). Deletion mutants were constructed that lack the amino terminal, the carboxyl terminal or both. Constructs VP6mt1-189 and VP6mt190-369 represent the amino and carboxyl halves of the full-length protein respectively. Truncated proteins VP6mt1-289 and VP6mt116-289 incorporate regions equivalent to domains 70 and 71 identified by Hayama and Li (1994). Truncated proteins VP6mt116-369 and VP6mt190-369 incorporate regions corresponding to all three domains (namely 70, 71 and 75).

Figure 4.8 summarizes the cloning strategy. Six truncated proteins were generated utilizing restriction endonuclease sites within the genome segment encoding VP6. The 5' modified VP6 gene of AHSV-6 cloned in the SP6 orientation of pGEM, was used as source plasmid for the generation of the truncated proteins. The full-length NS2 gene (segment 8, negative control) and the full-length VP6 genes (positive control) were digested out of their source plasmids using *Bam*HI and cloned into suitably prepared pFASTBAC Hta and Htb vectors respectively. AHSV NS2, which binds single-stranded RNA (Huismans *et al*., 1987a) but which does not bind dsRNA (Theron and Nel, 1997), was cloned and expressed for use as a control. Putative pFASTBAC Ht recombinants were screened using restriction endonucleases for insert and orientation. Recombinants were used for the generation of recombinant bacmids by transposition in DH10BAC cells. The equivalent positions of the regions identified as being important in dsRNA binding of BTV VP6 (regions 70,71 and 75) (Hayama and Li, 1994) are also indicated in figure 4.8. Of these, only region 75 is well conserved in different orbiviruses.

Composite bacmid DNA was screened for inserts by means of a standard PCR reaction using the polyhedrin promoter and the M13 reverse primers. A negative control containing no template DNA and a positive control using wildtype bacmid DNA as template were performed. Agarose gel electrophoresis showed the expected wildtype band in the order of 700bp (figure 4.9). The full-length NS2 and VP6 gene clones yielded the expected 1866 and 1852bp amplification products respectively. Clones for the expression of truncated proteins VP6mt1-115, VP6mt1-289, VP6mt1-189, VP6mt116-289, VP6mt116-369, and VP6mt190-369 yielded amplification products corresponding to the segment cloned plus the wildtype 700bp band namely: 1046bp;

1565bp; 1267bp, 1219bp; 1462bp and 1243bp respectively. Bacmids confirmed as positive recombinants using PCR were used for the transfection of Sf9 cells.

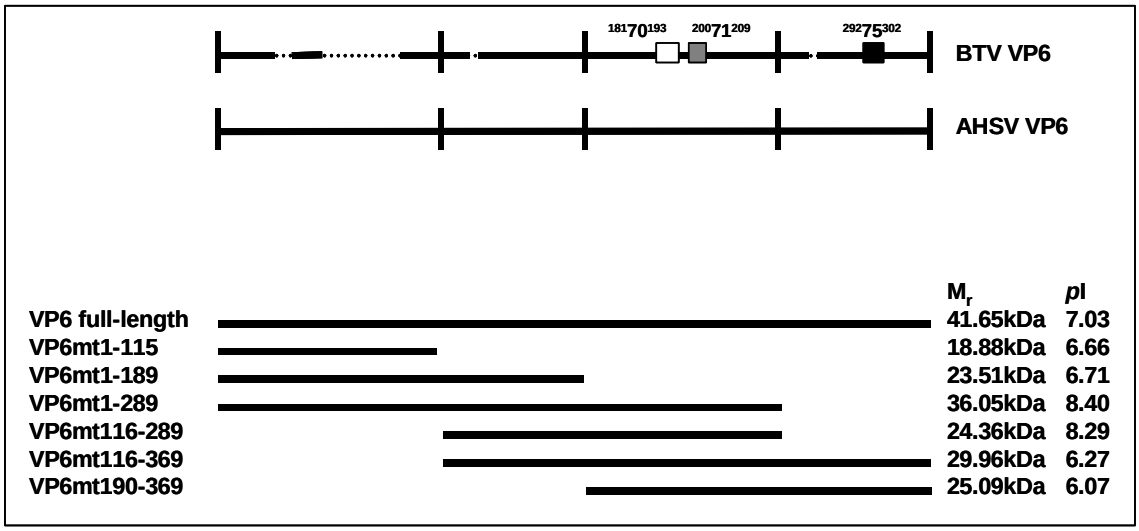

Figure 4.8 Schematic diagram illustrating the cloning strategy for the production of truncated VP6 peptides.

The positions of the binding domains identified on BTV VP6 (Hayama and Li, 1994) are indicated relative to AHSV VP6. Molecular weights and pI values were estimated for the expressed peptides including non-viral generated extensions using Compute pI/Mw (Bjellqvist *et al*., 1993; Bjellqvist *et al*., 1994; Wilkins *et al*., 1998) available through EXPASY (expert protein analysis system) hosted by the Swiss Institute of Bioinformatics.

4.3.9 EXPRESSION OF TRUNCATED PROTEINS IN A BACULOVIRUS SYSTEM AND IMMUNOLOGICAL SCREENING BY WESTERN BLOT

A series of truncated proteins with non-viral generated N-terminal (His-tag domain) and in some cases C-terminal extensions (fragments without the VP6 C-terminal) were expressed in *Spodoptera frugiperda* cells. Full-length VP6 and NS2 were used as positive and negative controls respectively. Figure 4.10a and b show the SDS-PAGE profile of the six truncated VP6 constructs prior to sucrose gradient purification. The truncated VP6 proteins expressed at comparatively similar levels. The proteins all exhibited slower electrophoretic mobility than predicted for the size of the genome segment encoding VP6 fragments and non-viral vector generated extensions. Both the full-length proteins of AHSV and BTV (Roy *et al.*, 1990) have been found to show slower electrophoretic mobility than predicted during SDS-PAGE. All the truncated VP6 proteins were found to be soluble and accordingly were partially purified by sucrose gradient fractionation. The truncated proteins were recovered from different fractions in the sucrose gradients in correspondence to the size of each peptide. There are, therefore, differences in the non-VP6 background proteins of the different VP6 peptides used in the binding assays. In a western immunoblot analysis, truncated proteins

VP6mt1-289, VP6mt116-289, VP6mt116-369 and VP6mt190-369 reacted positively to the anti-AHSV-3 antibodies (figure 4.10c). These truncated proteins have a region of amino acids from 191 – 289 in common. This result suggests a linear epitope within that region or a region exposed on the surface of the protein in its native conformation that is involved in immunological recognition.

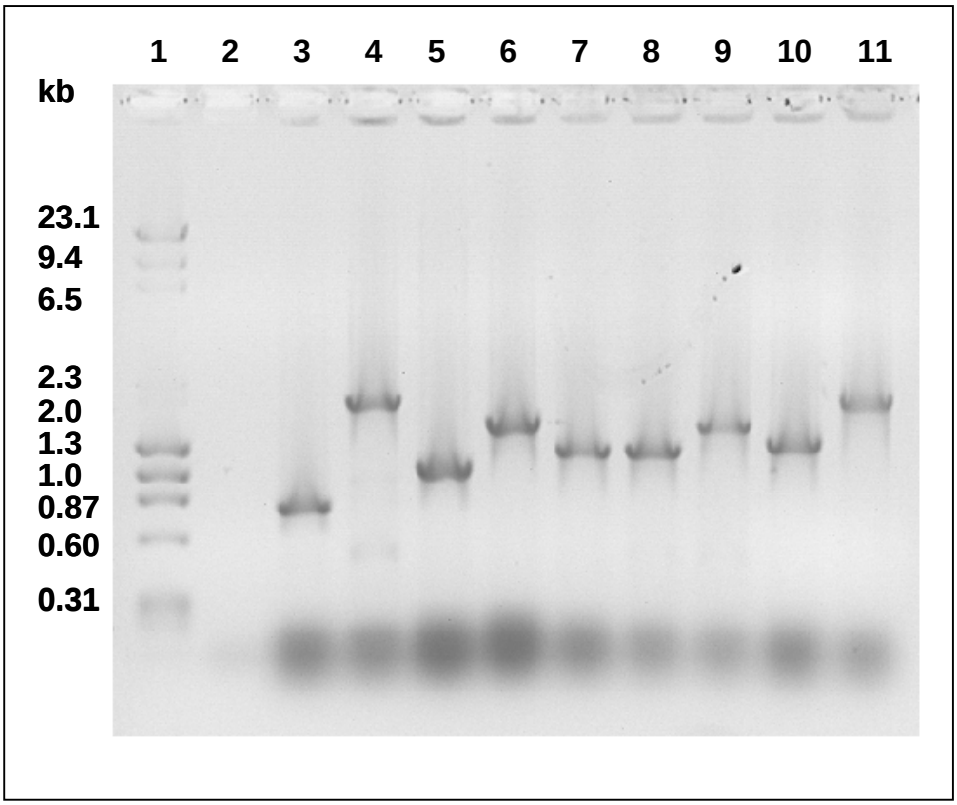

Figure 4.9 PCR screening of recombinant bacmid DNA used for transfection.

PCR products were separated by 1% 1 x TAE agarose gel electrophoresis.

Lane 1: DNA molecular weight marker II (Roche) and ϕX (Promega).

Lane 2: negative control (no template);

lane 3: wildtype bacmid DNA;

lane 4: VP6 recombinant;

lane 5: truncated VP6mt1-115;

lane 6: truncated VP6mt1-289;

lane 7: truncated VP6mt1-189;

lane 8: truncated VP6mt116-289;

lane 9: truncated VP6mt116-369;

lane 10: truncated VP6mt190-369 and

lane 11: NS2 recombinant bacmid.

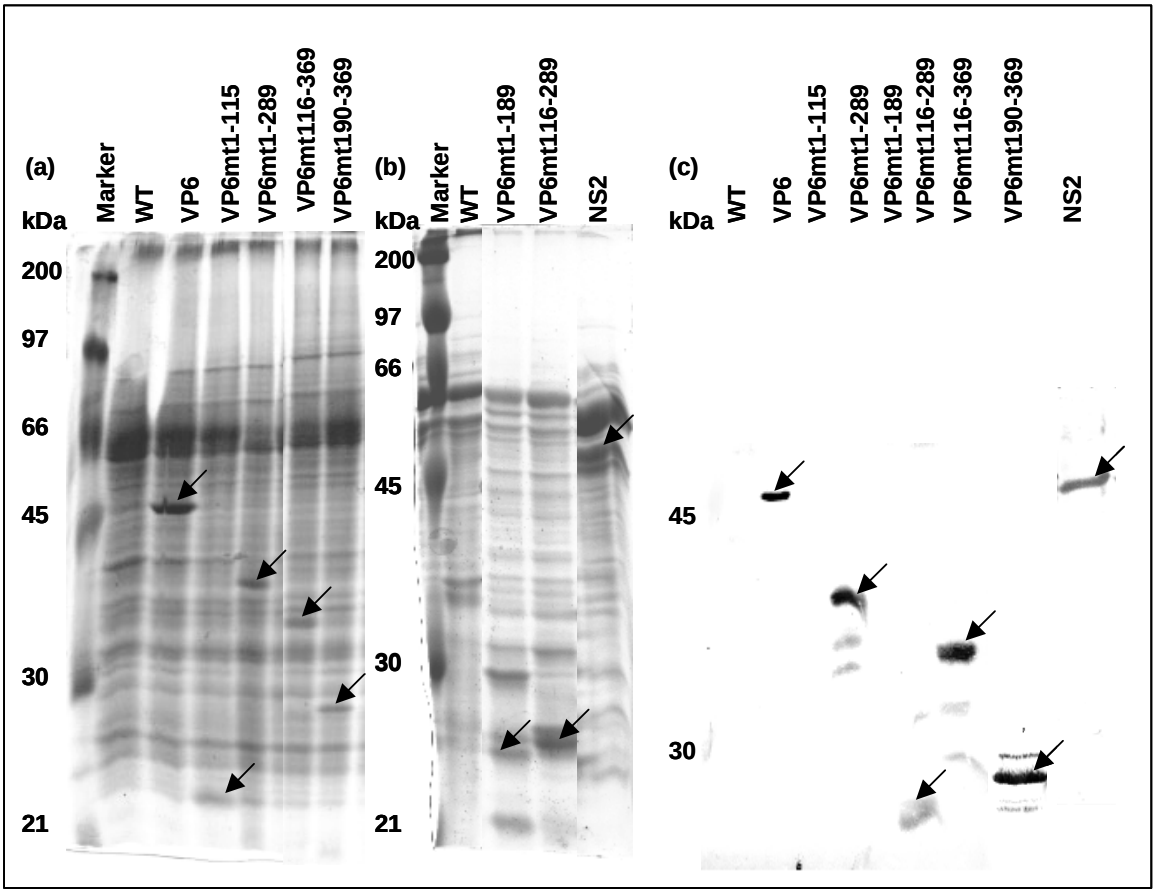

Figure 4.10 Expression and immunological screening of truncated proteins expressed in Sf9 cells.

(a and b) Coomassie stained SDS-PAGE analysis of the expression of truncated proteins in Sf9 cells by means of baculovirus recombinants prior to sucrose gradient purification. (c) Western immunoblot of truncated VP6 proteins using α-AHSV-3 guinea pig antiserum.

Fig 4.10(a):

lane 1: Rainbow marker;

lane 2: wildtype infected Sf9 cells;

lane 3: full-length VP6;

lanes 4 - 7: truncated VP6 proteins

Fig 4.10(b):

lane 1: Rainbow marker;

lane 2: wildtype infected Sf9 cells;

lanes 3 - 4: truncated VP6 proteins

lane 5: NS2 protein control

Fig 4.10(c):

lane 1: wildtype infected Sf9 cells;

lane 2: full-length VP6;

lanes 3 - 8: truncated VP6 proteins

lane 9: NS2 protein control

4.3.10 BINDING OF DOUBLE-STRANDED RNA BY TRUNCATED VP6 PROTEINS

Samples of sucrose gradient purified truncated proteins, were separated by SDS-PAGE, blotted onto a membrane and incubated in a 50mM NaCl standard binding buffer pH 7.0 in the presence of 1µg of labelled dsRNA (specific activity of 3 x 10^{10} cpm/µg). The membrane was washed as described in materials and methods (refer to section 4.2.1). Full length AHSV VP6 was used as a positive control and nonstructural protein NS2 of AHSV, which binds ssRNA, but not in a blot assay and does not bind dsRNA, was used as a negative control. Following autoradiography, it was evident that truncated proteins VP6mt1-289 and VP6mt116-289 bound the dsRNA (figure 4.11). The positive VP6 full-length control bound well and no binding was observed in the wildtype baculovirus infected Sf cells control or to AHSV NS2. The two VP6 mutants that bound the dsRNA probe (VP6mt1-289 and VP6mt116-289) have the region between amino acids 116 and 289 in common. However, this region is also present in VP6mt116-369 which does not bind dsRNA (figure 4.11b). There is also a partial overlap with VP6mt190-369 which also does not bind dsRNA. This result suggested that the addition of the C-terminal flanking region to truncated protein VP6mt116-289 eliminated binding of the protein. The amino acids in the C-terminal flanking region may have influenced the overall charge of the peptide.There may be specific domains involved in binding. These domains may have a highly positive charge. The VP6 binding domain could however, also be affected by the overall charge of the peptide, masking the effect of the smaller binding domain. The charge of the peptide is reflected by the isoelectric point. Isoelectric point (pI) estimations revealed that truncated proteins VP6mt1-289 and VP6mt116-289 have basic pI values of 8.4 and 8.29 respectively while truncated proteins VP6mt116-369 and VP6mt190-369 have acidic pI values of 6.27 and 6.07 respectively (figure 4.8). This raised the possibility that nucleic acid binding activity of VP6 assayed by nucleic acid overlay protein blot is strongly affected by differences in the net charge of the protein.

4.3.11 EFFECT OF pH ON DOUBLE-STRANDED RNA BINDING OF TRUNCATED VP6 PROTEINS

In order to investigate this further, the truncated protein binding assay was repeated with the truncated proteins above in 50mM NaCl SBB at a pH of 10.0 (figure 4.12a) and pH 6.0 (figure 4.12b). As expected, when the pH was raised above the estimated pI values of the proteins so that the proteins had a net negative charge, no binding was observed with the exception of some background in the wildtype control (figure 4.12a). When the pH was lowered to 6.0, the truncated proteins theoretically had a net positive charge and were induced to bind dsRNA. An increase in background was also observed at a lowered pH, therefore, the positions of the truncated proteins were verified using log graphs. The results appear to suggest a region on VP6 which contains domains that are important for dsRNA binding. This binding is abolished if the binding domain is linked to an amino acid sequence that lowers the pI. Binding to such a peptide can be

restored if the pH during the binding assay is lowered to give the peptide a net positive charge and this suggests that some level of binding specificity is maintained. From the results, it would appear that nucleic acid binding of AHSV VP6 can be manipulated by changing the binding assay conditions. This would suggest an important role for charge in the nucleic acid binding activity of the denatured VP6 protein of AHSV.

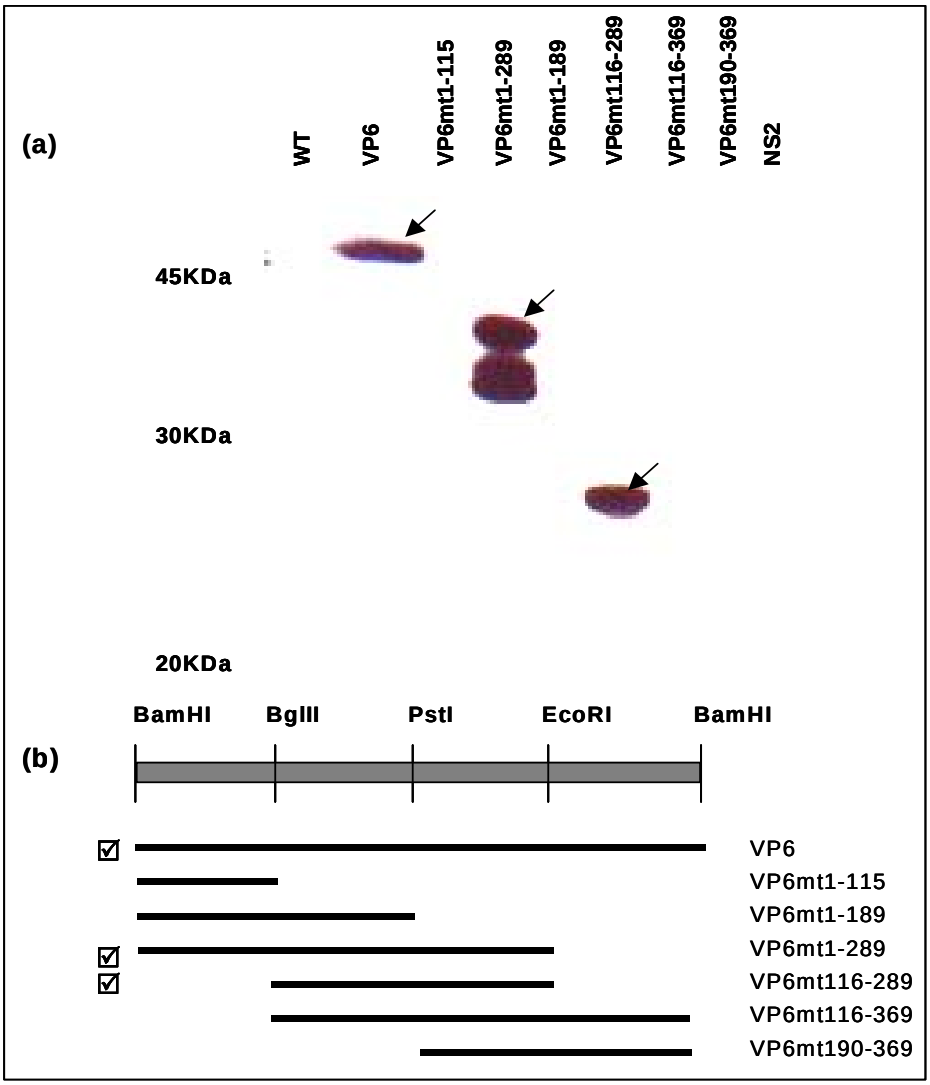

Figure 4.11 DsRNA binding by truncated VP6 proteins.

Autoradiogram of dsRNA binding activity of sucrose purified truncated AHSV-6 VP6 proteins assayed by nucleic acid overlay protein blots at pH 7.

Lane 1: wildtype baculovirus infected Sf cell proteins;

lane 2: full-length VP6 protein;

lane 3 - 8: truncated VP6 proteins;

lane 9: NS2 protein control.

Schematic diagram illustrating which truncated proteins bound dsRNA.

☑ indicates binding

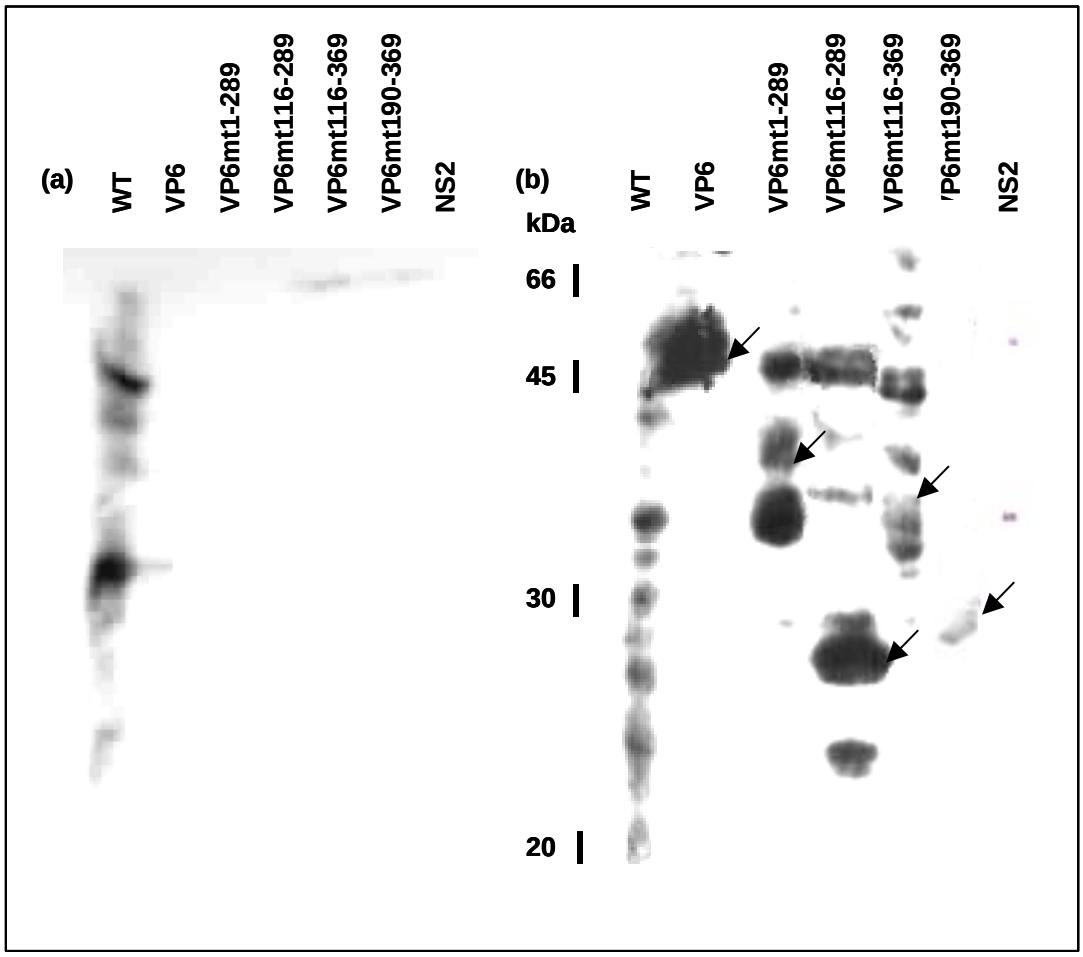

Figure 4.12 The effect of pH on dsRNA binding activity of the truncated AHSV-6 VP6 peptides.

Binding of sucrose purified truncated peptides was assayed by nucleic acid overlay protein blots at (a) pH 10.0 and at (b) pH 6.0. Positions of truncated proteins were verified using log graphs.

In both a) and b),

lane 1: wildtype baculovirus infected Sf cell proteins;

lane 2: full-length VP6;

lanes 3 – 6 : truncated VP6 proteins

lane 7: NS2 protein control.

4.3.12 DEMONSTRATION OF BINDING ACTIVITY OF BACTERIALLY EXPRESSED VP6 BY EMSA

Hayama and Li (1994), demonstrated the presence of domains required for the conformational binding of nucleic acids by BTV VP6 expressed in bacterial cells. A question remained as to whether the lack of binding of nucleic acids by bacterially expressed AHSV VP6 was due to a technical artefact or some aspect of the protein itself. In order to clarify this, partially purified bacterially expressed VP6 was used in an electrophoretic mobility shift assay (EMSA) using AHSV-6 dsRNA according to the method described by Hayama and Li (1994). Increasing amounts of VP6 protein from ±1μl to 4μl each of bacterial cell lysate expressing VP6 (as in figure 4.13a lane 3) were incubated with 125ng of AHSV dsRNA (segments 1 – 3 pool 1) followed by 0.8% agarose gel electrophoresis. Bacterially expressed VP6 was found to bind dsRNA as evidenced by an increased mobility shift with the addition of increasing amounts of protein (figure 4.13b). The controls of 1μl (lane 1) of bacterial cells (as in figure 4.13a lane 2); 20μl of bacterial cells (lane 5) and 1μg of BSA (lane 7) did not retard the mobility of the dsRNA (figure 4.13b). The problems experienced with attempts to demonstrate nucleic acid binding with bacterially expressed VP6 using a nucleic acid overlay protein blot can most probably be attributed to some aspect of the assay, most probably the blotting of the proteins to the membrane which may be influenced by the blotting conditions.

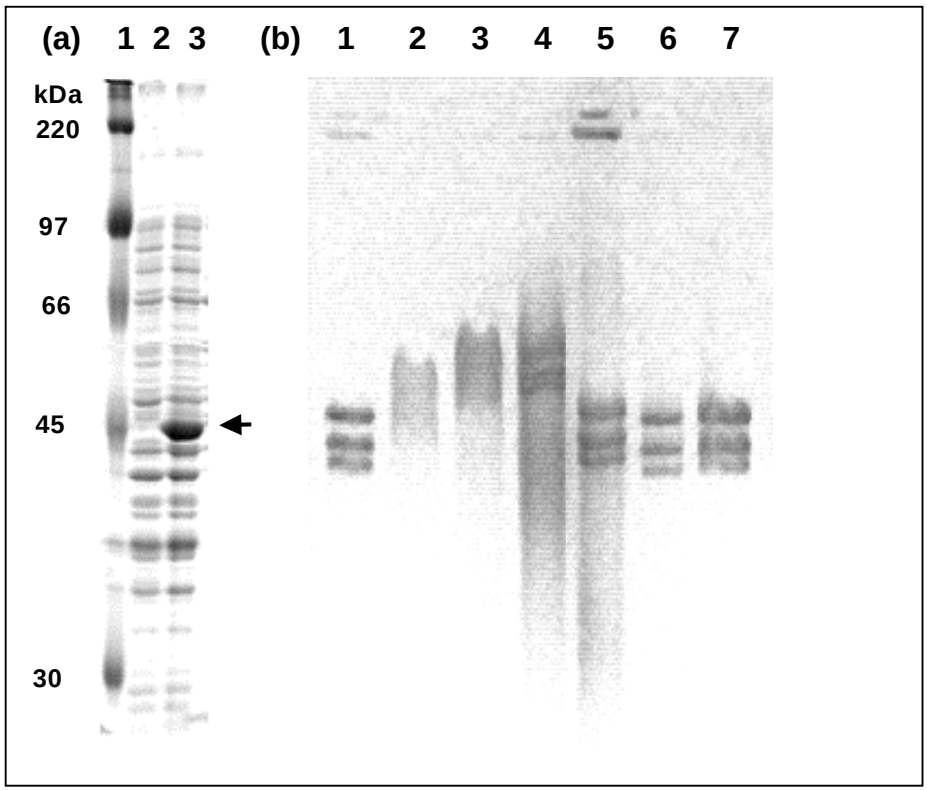

Figure 4.13 Nucleic acid binding activity of bacterially expressed AHSV-6 VP6.

(a) SDS-PAGE separation of bacterially expressed proteins. (b) Nucleic acid binding activity of bacterially expressed VP6 determined by electrophoretic mobility shift assay (EMSA).

(a) Coomassie blue stained SDS-PAGE gel of bacterially expressed proteins.

Lane 1: Rainbow marker;

lane 2: bacterial cells and

lane 3: VP6 expressed in bacterial cells.

The arrow indicates the VP6 protein.

(b) An electrophoretic mobility shift assay (EMSA) of 125ng of AHSV-6 dsRNA segments 1 – 3 (pool 1) incubated with increasing amounts of bacterially expressed VP6 protein and analyzed on a 0.8% TAE agarose gel.

Lane 1: dsRNA incubated with1μl of bacterial cell lysate;

lane 2 - 4: dsRNA incubated with 1μl, 2μl and 4μl of bacterial cell lysate expressing VP6 (±1 μg/μl) respectively.

lane 5: dsRNA incubated with an excess of 20μl of bacterial cell lysate without VP6;

lane 6: AHSV-6 dsRNA control and

lane 7: dsRNA incubated with 1μg of BSA.

4.4 DISCUSSION

The function of BTV VP6 has been investigated by various research groups. Aspects of nucleic acid binding activity have been elucidated and it has been demonstrated that BTV VP6 has ATPase linked helicase activity (Roy *et al.*, 1990; Hayama and Li, 1994; Stäuber *et al*., 1997). Based on sequence similarity, the equivalent protein in AHSV is considered to be the putative helicase for the virus. As a dsRNA helicase, VP6 could be expected to bind both single-stranded and double-stranded RNA (Lohman and Bjornson, 1996). As yet, no functional analyses have been performed on the AHSV VP6 protein. The objective of the work described in this chapter was the demonstration and characterization of nucleic acid binding activity of VP6 with the aim of proposing a mechanism responsible for this activity. In order to address this aim, the initial step was to demonstrate nucleic acid binding activity.

Nucleic acid binding was demonstrated by means of nucleic acid overlay protein blots or north western assays as described by Roy *et al*. (1990) and Mears and Rice (1996). Roy *et al.* (1990) successfully used BTV VP6 that had been partially purified by sucrose gradient fractionation for nucleic acid overlay protein blot assays. Accordingly, all further binding studies were performed using partially purified proteins which were prepared by sucrose gradient fractionation.

Baculovirus expressed AHSV VP6 was demonstrated to bind dsRNA by nucleic acid overlay protein blot assays. Bacterially expressed VP6 did not bind dsRNA in the same assay. Some difficulty was previously identified during western blotting of bacterially expressed VP6 which also relies on transfer of proteins to a nitrocellulose membrane. No valid reason could be identified to offer an explanation for the lack of blotting of the bacterially expressed VP6 protein to the membrane. Differences in two characteristics of AHSV VP6 expressed in a baculovirus and a bacterial system that may influence binding activity have been identified. Firstly, bacterially expressed VP6 appears to have a higher sedimentation value than baculovirus expressed VP6 (refer to section 3.3.5). This may indicate different oligomeric structures or different protein folding for VP6 expressed in the two systems. These differences should, however, be eliminated during SDS-PAGE. Secondly, it has been demonstrated that the baculovirus expressed VP6 is glycosylated (refer to section 3.3.7). Both possibilities were evaluated but characterization of AHSV VP6 nucleic acid binding was focussed on baculovirus expressed VP6.

Having demonstrated that AHSV VP6 binds dsRNA, the next objective was to evaluate whether baculovirus expressed AHSV VP6 could bind various types of nucleic acid by means of a north western binding assay. Each type of nucleic acid was labelled in a different manner. The specific activities were calculated to be in the order of 10^6cpm/μg for ssRNA, and DNA probes. The dsRNA probe was calculated to have a specific activity of approximately 10^{10}cpm/μg. AHSV VP6 was found to bind AHSV and BTV

dsRNA as well as virus specific ssRNA generated by *in vitro* transcription. VP6 was also found to bind both single-stranded and dsDNA. It seems, therefore, that unlike NS2 of BTV (Huismans *et al.*, 1987a), EHDV (Theron *et al*., 1994) and AHSV (Uitenweerde *et al*., 1995) which only bind ssRNA, VP6 is capable of binding all four types of nucleic acid including dsRNA from BTV. This corresponds with evidence for nucleic acid binding activity of BTV VP6 presented by Roy *et al*. (1990) where it was demonstrated to bind single-stranded and double-stranded RNA and DNA.

In order to characterize nucleic acid binding activity, the effect of various salt concentrations was assayed. Salt concentrations of 50mM through to a higher concentration of 150mM were used for this assay. With all four types of nucleic acid probes, a continuous decrease in binding was observed as the salt concentration increased. SsDNA bound least efficiently with less nucleic acid bound at the onset (50mM NaCl) and no binding by 125mM NaCl. VP6 nucleic acid binding activity is strongly affected by an increase in salt concentration. This may be because the increase in ion concentration changes the net charge on the proteins and nucleic acids involved and directly influences the level of electrostatic binding.

As a putative dsRNA virus helicase, VP6 may bind single-stranded and dsRNA and could exhibit a preference for RNA over DNA. In order to investigate this hypothesis, competition assays were performed. Competition assays were used by Lemay and Danis (1994) to establish nucleic acid affinity for the reovirus λ1 protein. Finerty and Bass (1997), successfully applied competition assays to establish dsRNA binding by a zinc finger protein of *Xenopus*. Labelled ssRNA and dsRNA were prepared and the specific activities determined. A fixed amount of VP6 was then probed with increasing amounts of the labelled nucleic acid until a saturation point for the binding of 1μg of VP6 to ssRNA and dsRNA was established. In the first round of competition assays, labelled ssRNA was used as probe. DNA (both single and double-stranded) proved a poor competitor to ssRNA binding. Although the addition of dsRNA as competition agent caused an initially larger decrease in binding of the ssRNA probe, both single and double-stranded RNA resulted in a decrease in the order of 62 – 63% following the addition of 4x the unlabelled competition nucleic acid. These results suggest that VP6 has a greater affinity for RNA than DNA. In order to determine whether a preference for single or double-stranded RNA could be established, competition assays using a labelled dsRNA probe were performed. Initially, unlabelled dsRNA resulted in a greater decrease in binding of the dsRNA probe. After the addition of 4x the unlabelled competition nucleic acid, dsRNA decreased the binding by 6% more than ssRNA. This may indicate a slight preference for VP6 binding of dsRNA over ssRNA. A dsRNA helicase may bind both single-stranded and double-stranded regions at an unwinding junction depending on the mechanism of helicase action (refer to section 1.10.1). AHSV VP6 binds both ssRNA and dsRNA more efficiently than it binds DNA. This is in line with what would be expected of a RNA helicase.

It is thought that binding and dissociation of hnRNPs from RNAs and other proteins is regulated. It has been suggested that such interactions may be controlled by post-translational modification as most hnRNPs have been found to be post-translationally modified. These modifications include methylation, phosphorylation and glycosylation (Liu and Dreyfuss, 1995). N-linked glycoproteins are thought to play a role in among other things, recognition and regulation of protein receptors (Elbein, 1987). The first glycosylated RNA binding protein, P43 was described by Soulard *et al*. (1993). These authors speculated on a possible role for glycosylation in the function of this protein. As N-glycosylation is a feature of eukaryotes and eukaryotic virus proteins, it was deemed worthy of investigation. N-glycosylation would not be expected and was not found in bacterially expressed VP6. Studies using tunicamycin to evaluate the role of carbohydrate have revealed that the only unifying concept to explain all situations is that the addition of a carbohydrate affects the conformation of a protein. The oligosaccharide may affect the stability of the protein with regard to denaturation and proteolysis; may increase the solubility of the protein; cause the exposure of sites that involve transport, membrane interaction and / or function of the protein and may play a role in biological recognition on its own (Elbein, 1987). The glycosylation of VP6 may well be an artefact of the baculovirus expression system and be triggered by the known glycosylation motif that occurs at residues 48-51 on VP6. However, the glycosylation of the baculovirus expressed protein could have affected the dsRNA binding studies. For that reason, glycosylated and deglycosylated VP6 protein were compared with respect to dsRNA binding. N-Glycosidase F and tunicamycin treatment were used to obtain deglycosylated baculovirus expressed VP6 protein. Unsatisfactory results were obtained using N-Glycosidase F to deglycosylate VP6. In general, the baculovirus expressed protein was found to be fairly unstable and very susceptible to degradation following freeze-thawing. In comparison, the bacterially expressed VP6 and tunicamycin treated VP6 (to a lesser extent) were found to be more robust and resistant to the effects of freeze-thawing. This may be as a result of the N-glycosylation and its effect on the stability of the protein. Elbein (1987), suggested that tunicamycin may affect the synthesis and decrease the solubility of a protein. As a result, these two aspects were assessed. A relatively low concentration of tunicamycin was sufficient for inhibiting glycosylation of VP6 in Sf9 cells. Even as much as 10μg/ml had no negative effects on protein synthesis. No apparent change in solubility was observed in deglycosylated VP6 arising from tunicamycin treatment. A western blot revealed that immunological recognition of VP6 is not determined by the presence of a N-linked glycan. Tunicamycin deglycosylated VP6 retained its dsRNA binding activities. It would appear that glycosylation is not integral to the binding activity of AHSV VP6.

Hayama and Li (1994), used truncated deletion mutants to map and characterize antigenic epitopes and nucleic acid binding domains of BTV VP6. Truncated deletion mutants are frequently used for the purpose of identifying linear epitopes involved in nucleic acid binding (Brantley and Hunt, 1993; Labbé *et al*., 1994; Lemay and Danis, 1994; Fernández *et al*., 1995; Mears *et al*., 1995; Bleykasten *et al*., 1996; Fernández

and Garcia, 1996; Mears and Rice, 1996; Wang *et al*., 1996b). Numerous common motifs that are important for nucleic acid binding have been identified (Mattaj, 1993; Burd and Dreyfuss, 1994; Sachetto-Martins *et al*., 2000). Glycine rich nucleic acid binding proteins with specific consensus sequences have been identified. These sequences are usually found in conjunction with other binding motifs such as the dsRBDs or RNP sites (Sato, 1994; Zhang and Grosse, 1997; Sachetto-Martins *et al*., 2000). As is evident from chapter 2, there are few conserved sequences in VP6 that have been associated with nucleic acid binding. When AHSV; BTV, Chuzan and St Croix River virus VP6 proteins were compared (figure 2.11), with respect to regions important for binding identified by Hayama and Li (1994) for BTV VP6, the following was observed. Epitope/binding domain 70 is not conserved in AHSV; domain 71 shows some level of conservation and domain 75 is well conserved in the four *Orbivirus* VP6 proteins compared. Based on primary structure of the protein, there were no clear target areas for the development of truncated proteins. As a result a “shotgun” approach was followed whereby fragments representing sections of the gene were cloned and expressed. This incorporated the equivalent regions in AHSV VP6 that correspond with Hayama and Li’s domains (1994). The truncated proteins were used to investigate whether regions containing linear epitopes for immunological recognition and domains involved in nucleic acid binding activity could be identified. Western immunoblot analysis indicated a region of 98 amino acids between position 191 and 289 (from the first methionine) which may be important in immunological recognition. From the 6 overlapping truncated VP6 proteins (Fig 4.8), only two (VP6mt1-289 and VP6mt116-289) bound the dsRNA probe, whereas a third peptide (VP6mt116-369) which overlaps a shared region and a partially overlapping fourth peptide (VP6mt190-369), did not bind. The proteins which did not bind, effectively acted as negative controls for the involvement of the 6his tag in binding. However, in order to eliminate any possible involvement of the 6his tag, the binding experiment was also performed with the fragments for expression of truncated proteins VP6mt1-115; VP6mt1-289 and VP6mt1-189, which have the 5’ end intact and thus provide their own start codon, cloned into pFASTBAC (no 6his tag). The results were identical. The differences in binding of the truncated peptides could be explained by comparing the isoelectric points of the respective truncated proteins.

Molecules containing both acidic and basic groups are known as ampholytes. Proteins which are large molecules with many acidic and basic groups are called polyampholytes. At a particular pH the acidic and basic charges will cancel each other out and the net charge will be zero. This is the isoelectric point (pI) of the protein (Dunbar, 1987). If the majority of charged groups are acidic, the isoelectric point will be low (<7), while if basic groups predominate, the pI will be high (>7) (Mathews and van Holde, 1991). Predominantly acidic proteins have a negative charge at a neutral pH while predominantly basic proteins have a positive charge at a neutral pH (Campbell, 1995). Therefore, if a protein is in an environment with a pH higher than its pI, it will have a net negative charge while, at a pH lower than its pI, it will have a net positive

charge. The effects of pH are important in protein chemistry. It has been found that a small shift in pH may change the constellation of charges on a protein molecule to the extent that its behaviour may be significantly modified (Mathews and van Holde, 1991).

Peptides that did bind dsRNA had basic pIs of above 8 while VP6mt116-369 and VP6mt190-369 have predicted acidic pI values of just above 6. All four of the truncated proteins under consideration share an amino acid overlap from residue 190 to 289. The difference in pI values can be accounted for by the addition of the acidic C-terminal to the truncated proteins that bind dsRNA at pH 7. This region is rich in acidic residues and has a predicted pI of 5.04. The addition of this acidic 80 amino acid region to a basic or neutral peptide results in a change in the protein parameters of the truncated proteins and may affect binding activity because the total charge of the peptide has changed. This suggested that pH could play an important role in determining which peptides were likely to bind and which not. If a protein is in an environment with a pH higher than its pI, it will have a net negative charge while, at a pH lower than its pI, it will have a net positive charge. In order to investigate, the binding assay was carried out at a pH of 6.0 at which the peptides are assumed to have a largely positive charge and at pH 10.0 where they would have a net negative charge. When the pH was raised to 10, all binding activity was eliminated barring some background in the wildtype control. When the pH was lowered to 6, it would appear that binding could be induced by neutralizing the effect of the acidic region of amino acids joined to a basic or neutral peptide containing the AHSV VP6 binding domain.

Similar to BTV VP6, the VP6 protein of AHSV has numerous basic residues distributed throughout the protein. In the case of BTV VP6, it has the highest number of charged residues per unit length of 1000 amino acids (Fukusho *et al*., 1989). This number is comparable to what has been observed in AHSV VP6. It may be postulated that the dsRNA binding domain in AHSV VP6 is not a rigid consensus sequence of amino acid residues, but rather a sequence of positively charged amino acids that constitute a domain that determines the nucleic acid binding characteristics of the peptide. If a predominantly negatively charged peptide is added to this region, binding activity is abolished. Binding can be reestablished by manipulating the pH of the binding conditions. Peptides lacking the binding domain, show no binding activity at all regardless of the binding assay conditions. The regions on AHSV VP6 that corresponded to BTV VP6 binding domains 70 and 71 (Hayama and Li, 1994) were located between amino acid residues 190 to 289. The binding domains identified on BTV VP6 (Hayama and Li, 1994) are characterized by a high number of positively charged amino acid residues. When the overlapping region of the peptides which did bind was analyzed, two regions rich in basic residues were identified.

The effects of increasing salt concentration support the role of charge as the mechanism of binding. It has been demonstrated that salt concentration and pH have a marked effect on the nucleic acid binding activity of AHSV VP6. Similar results were obtained for the RNA binding domain of Southern cowpea mosaic virus (SCPMV) (Lee

and Hacker, 2001). In SCPMV, the binding domain has a number of basic residues, none of which are specifically required for RNA binding. Binding activity is determined by the overall charge of the domain. Electrostatic interactions are thought to be important for binding. Binding due to charge may erroneously be ascribed to an alternative domain specific mechanism, unless the pI of the peptide as well as the salt and pH effect on binding are taken into account.

Hayama and Li (1994), proposed that the nucleic acid binding domain in BTV may be single but conformational. They found that deletion of amino acid residues 292 – 302 (epitope 75) did not affect binding activity. This is the only epitope of the three that shows high levels of conservation in AHSV VP6. These authors found that deletion of the amino acid residues corresponding to domain 71 eliminated binding activity. This region is not well conserved in AHSV VP6. They further proposed that the three domains 70; 71 and 75, when juxtaposed, form a conformational domain that enables nucleic acid binding. These three domains are described as being rich in arginine and lysine which suggests a positive net charge. Six epitopes were identified by Hayama and Li (1994), generated as peptides and tested for binding activity. Of these, three bound nucleic acids and three did not. As a matter of interest, using the information supplied by Hayama and Li (1994), the isoelectric points of the six peptides representing six epitopes in BTV were estimated using Compute pI/Mw (Bjellqvist *et al*., 1993; Bjellqvist *et al*., 1994; Wilkins *et al*., 1998) available through EXPASY (expert protein analysis system) hosted by the Swiss Institute of Bioinformatics. The results are summarized in table 4.1.

Table 4.1 Estimated pI values of six epitopes identified in BTV VP6.

Epitope[a]	Bound nucleic acids[a]☑	Estimated pI[b]	Residue position in BTV VP6[a]	Peptide sequence[a]
72	☒	6.29	35 - 46	KENKTEPKEESK
74	☒	5.00	63 - 78	KEEGGKETKDADVDRR
70	☑	9.97	181 - 193	ERLRDLRRKEKSE
71	☑	10.9	200 - 209	ERGGRKQRKE
73	☒	4.37	216 - 227	REGVEEEKTSEE
75	☑	10.0	292 - 302	KEVAREASKKK

(a) Hayama and Li (1994); b) Compute pI/Mw (Bjellqvist *et al*., 1993; Bjellqvist *et al*., 1994; Wilkins *et al*., 1998).

BTV VP6 nucleic acid binding studies using EMSA were performed at pH 7.5 (Hayama and Li, 1994). Of the six domains identified in BTV VP6, three have basic isoelectric points and will have a net positive charge at pH 7.5. Peptides representing these same three domains i.e. 70; 71 and 75 bound nucleic acids. The remaining three have acidic

pIs resulting in a net negative charge at pH 7.5. Peptides representing these three epitopes i.e. 72; 74 and 73 did not bind nucleic acids. It is, therefore, possible that charge plays a role in nucleic acid binding in BTV VP6. Total amino acid composition (and resulting pI) of a binding domain, may in this instance be more important than its actual sequence.

N-glycosylation may play a role in the regulation of VP6 protein function by affecting the pI of the protein. Glycosyl phosphatidylinositol-anchored proteins have multiple isoforms with different isoelectric points which are consistent with extensive but differential N-glycosylation. GPI-anchored proteins with a lower M_r had a more basic pI. Therefore, the more heavily N-glycosylated a protein, the more acidic its pI (Fivaz *et al*., 2000).

The final question that was addressed was the failure of bacterially expressed VP6 to bind nucleic acids in a nucleic acid overlay protein blot assay. Having established that N-glycosylation played no direct role in the nucleic acid binding activity of VP6, the possibility remained that some artefact, possibly in the process of blotting, had resulted in the lack of binding. To avoid blotting, electrophoretic mobility shift assays were performed as described by Hayama and Li (1994). Using AHSV dsRNA segments 1 – 3 (pool 1), bacterially expressed VP6 bound the dsRNA as evidenced by the retardation of its movement through the agarose gel. Controls, of bacterial cell lysate which did not contain VP6 and BSA, did not bind the dsRNA. It appears that concentration plays a role as was described for BTV VP6 (Hayama and Li, 1994). It, therefore, seems fairly evident that the lack of binding of bacterially expressed VP6 in nucleic acid overlay protein blot assays was due to an artefact introduced during the blotting process and not a lack of binding activity *per se*.

CHAPTER 5:CONCLUDING REMARKS

The broad aim of this study was to characterize the structure and nucleic acid binding function of AHSV VP6. A number of short term aims were directed at addressing important questions such as: whether there is evidence suggesting that VP6 is the AHSV helicase; whether there are any specific motifs in the amino acid sequence which would suggest its function within the virus. What properties does the protein exhibit i.e. is it soluble, is there evidence of post- or co-translational modification. Whether there is any evidence that VP6 binds nucleic acids and if so, by what mechanism. Is there sequence specificity or is binding only as a result of charge? Whether there is a difference in the binding of ssRNA, dsRNA and DNA.

Analysis of the primary structure showed the glycine rich nature of AHSV VP6. This is in agreement with other *Orbivirus* VP6 sequences. Variation between serotypes is low and between serogroups is high.

Double-stranded RNA helicases should theoretically demonstrate some homology. The homology of VP6 in terms of available *Orbivirus* sequences was investigated. Exhaustive maximum parsimony methods applied to the nucleotide data yielded a single tree in which BTV and AHSV VP6 genes grouped together with a boodstrap value of 100. The results of functional constraint analysis were inconclusive as different results were obtained depending on the treatment of the gaps. In many protein alignments, gaps would not be as relevant, however, in the VP6 alignment gaps play a prominent role. As a result of the difference in length of the various *Orbivirus* VP6 genes and/or proteins, their value in phylogenetic analyses is questionable.

Analysis of the primary structure identified motifs common to glycine rich proteins. As mentioned above, in biting midge borne *Orbivirus* VP6 proteins investigated to date, the most prevalent amino acid in terms of amino acid content is glycine. Various glycine motifs common to glycine rich proteins have also been identified.

The positions of three domains identified in BTV VP6 thought to be important for binding (Hayama and Li, 1994) were located on AHSV VP6. These authors proposed that BTV VP6 bound nucleic acids in a conformational manner as a result of the juxtapositioning of these three epitopes. Only one of these epitopes showed conservation between AHSV and BTV VP6 proteins. These regions are, however, characterized by between 55 to 60% basic amino acid residues.

Regions associated with helicase activity such as a conserved region found in the Rep helicase of *E. coli*, the “A” and “B” sites of the Walker box involved in ATP binding and hydrolysis as well as two motifs common to SF2 helicases were identified. From the primary structure analysis it may be predicted that VP6 of AHSV is the most likely candidate for helicase activity. Several post translational modification signals were identified, including a N-glycosylation signal. Hydrophillicity plots predicted a soluble

protein. Specific secondary structures characteristic of nucleic acid binding proteins were not identified in AHSV VP6.

In order to function as a helicase, AHSV VP6 must bind and hydrolyse ATP for energy. In order to unwind double-stranded nucleic acid, it must bind nucleic acids. Numerous amino acid sequences or motifs have been associated with nucleic acid binding. In BTV VP6, Kar and Roy (2003), have shown the importance of an arginine residue in a highly positively charged domain that is similar to the conserved helicase motif VI. This domain is not conserved in AHSV VP6, Chuzan virus VP6 or St Croix River virus VP6. No clear indication of nucleic acid binding motifs with the exception of generally distributed glycine rich motifs were identified in AHSV VP6.

Evidence of nucleic acid binding by AHSV VP6 was sought using baculovirus and bacterially expressed VP6. Partially purified proteins were successfully applied in a RNA overlay protein blot binding assay for the demonstration of dsRNA binding of baculovirus expressed VP6. Bacterially expressed VP6 did not bind the dsRNA probe in this assay. There are two major differences between baculovirus and bacterially expressed VP6 protein. The first of those is that glycosylation of baculovirus expressed VP6 is not found in bacterially expressed VP6. The other difference is an increase in the sedimentation value of bacterially expressed VP6 as compared to baculovirus expressed VP6. This suggests a difference in either folding or oligomerization of the bacterially expressed protein.

In order to characterize the nucleic acid binding activity of AHSV VP6, a number of questions were addressed. Based on the findings of Roy *et al*. (1990), the first question was whether VP6 has an affinity for different types of nucleic acids. It was established by north western assay that AHSV VP6 binds both single and double-stranded RNA and DNA of AHSV origin as well as dsRNA of BTV. In the electrophoretic mobility shift assays (EMSA) performed, bacterially expressed VP6 bound both viral specific and non-specific DNA as well as dsRNA.

The basic amino acid content of AHSV VP6 suggested a possible role for charge in the nucleic acid binding mechanism of the protein. Accordingly, the effect of salt concentration on the affinity of AHSV VP6 for nucleic acids was determined. VP6 was assayed for binding activity with all four different AHSV specific nucleic acid species at increasing salt concentrations. As salt concentration increased, the affinity of VP6 for each nucleic acid decreased. Due to the effect of salt in the electrostatic screening of nucleic acids and proteins, this result supports a role for charge in the nucleic acid binding mechanism of VP6.

Most models proposed for helicase activity assume that the helicase should be capable of binding to both single and double-stranded nucleic acids. The hypothesis was, therefore, proposed that AHSV VP6 as the putative helicase of a dsRNA virus, should preferentially bind RNA as opposed to DNA. This was demonstrated by competition assays with a radioactively labelled ssRNA probe. An attempt to establish a preference

between single and double-stranded RNA was made using a radioactively labelled dsRNA probe. The results suggested that VP6 may exhibit a slight preference for binding dsRNA over ssRNA.

Although the glycosylation of baculovirus expressed VP6 may be an artefact of the expression system, it could have affected the dsRNA binding studies. Therefore, the role of N-glycosylation in immunological recognition and binding ability of AHSV VP6 was investigated. Following tunicamycin treatment to inhibit glycosylation of VP6 expressed in insect cells, a western immunoblot revealed that immunological recognition is not determined by the presence of a N-linked glycan. A dsRNA overlay protein blot assay demonstrated that baculovirus expressed VP6 treated with tunicamycin retained its affinity for dsRNA. N-glycosylation may play a role in the regulation of VP6 protein function as N-glycosylation lowers the isoelectric point (pI) of a protein (Fivaz *et al*., 2000).

To investigate regions that may be important in nucleic acid binding activity, deletion mutation analyses were performed. Following the analysis of AHSV VP6 aligned to BTV VP6, a series of truncated proteins were generated which accommodated regions of AHSV VP6 which correspond to the three epitopes identified by Hayama and Li (1994). The results suggested that a specific sequence of amino acids was not the determining factor for nucleic acid binding as truncated proteins with the same overlap did not necessarily all bind. One major difference that could explain the differences in binding is the isoelectric points of the respective truncated proteins.

The hypothesis was, therefore, proposed that binding of denatured AHSV VP6 in a RNA overlay protein blot binding assay is as a result of charge. This hypothesis was tested by repeating the assays at pH 10 where the proteins should have a net negative charge, and pH 6 where the proteins should have a net positive charge. It may be proposed that the nucleic acid binding activity of AHSV VP6 is determined by a series of positively charged amino acids (localized between residues 190 and 289) that constitute a domain that determines the nucleic acid binding characteristics of the peptide. The results suggest a role for electrostatic binding in non-conformational nucleic acid binding activity of AHSV VP6. These results were supported by the effect of increasing concentrations of NaCl on the nucleic acid binding affinity of VP6.

Amino acid regions predicted to bind did not necessarily bind. A stretch of amino acids may be predicted to bind based on amino acid composition. However, if it is attached to a region rich in acidic (negatively charged) amino acids it no longer binds nucleic acids. Binding can be reestablished by manipulating the pH of the binding conditions. Peptides which do not contain the binding domain, show no binding activity at all regardless of the binding assay conditions. An argument could be made for the use of smaller peptides. However, even if smaller peptides are used, the basic amino acid content and resulting pI of the peptide would still be relevant to its capacity for binding nucleic acids. It is as a result of the use of overlapping truncated proteins that the charge effect was identified. Theoretically, if a region rich in basic amino acids was removed from a

protein, the rest of the protein may not bind nucleic acids as the amino acid content of the peptide has changed. On the other hand, a strongly acidic residue containing peptide added to a peptide shown to bind nucleic acids may cause the resulting peptide to no longer bind. Truncated protein VP6mt116-289 used in this study (172 amino acids) has a theoretical pI of 8.29 and has been demonstrated to bind nucleic acids at pH 7. Truncated protein VP6mt116-369 has an additional peptide of 80 amino acids added to truncated protein VP6mt116-289. The additional 80 peptide region has a predicted pI of 5.04. The predicted pI for truncated protein VP6mt116-369 (total of 254 amino acids) is 6.27 which means that it has an overall negative charge at pH 7. Truncated protein VP6mt116-369 does not bind nucleic acids at pH 7. The addition of the acidic residues in the 80 amino acid peptide has changed the character of the peptide by changing the charged amino acid content resulting in a loss of binding activity.

In order for a helicase to get near to the nucleic acid to be unwound, it must be basic as like charges will repel each other. The evidence presented in this study suggests that the manner in which AHSV VP6 binds nucleic acids necessary for its function as a helicase, is related to charge. Traditionally, it has been proposed that a specific “motif” or sequence of amino acids confers binding activity to the protein. If changes or mutations are introduced into that motif, binding activity is abolished. It may in fact be that the mutations are changing the character of the entire peptide and as a result of these changes, the isoelectric point changes. The overall charge of the protein at that pH changes, and the protein no longer binds nucleic acids. Therefore, total amino acid composition (and resulting pI) of the binding domains may be of more importance than the actual sequence of amino acids.

Most authors report binding studies performed at 50mM salt concentration and a neutral pH (pH 7.0 – 7.5). These results have shown that if either of these conditions are changed, the results are very different.

Bacterially expressed VP6 was demonstrated to bind dsRNA in an electrophoretic mobility shift assay (EMSA) as described by Hayama and Li (1994). These authors reported that nucleic acid binding was concentration dependent. Partial purification raised a potential problem with EMSA as concentration can only be estimated.

For further investigations of the functions of AHSV VP6 one could include an investigation into the role of the two regions rich in basic residues found in the region of amino acid residues from 190 – 289 which appears to be important in nucleic acid binding; a mechanism for specific nucleic acid binding; for ATP binding and hydrolysis and helicase activity, highly purified protein is necessary. A system such as the baculovirus system which results in the expression of native proteins with post-translational modifications, correct folding and conformation is required. However, another vector system supplying an alternative method of purification is desirable.

CHAPTER 6:RESEARCH OUTPUT

Refereed Journals:	**Turnbull, P.J.**, Cormack, S.B. and Huismans, H. 1996. Characterization of the gene encoding core protein VP6 of two African Horsesickness virus serotypes. J. Gen. Virol. 77, 1421 – 1423. **De Waal P.J.** and Huismans, H. Characterization of the nucleic acid binding activity of inner core protein VP6 of African horsesickness virus. Accepted on 4 April 2005 for publication in Archives of Virology.
International Congress Participation:	Cormack, S.B., **Turnbull, P.J.** and Huismans, H. (1995) Characterization of the Inner Core Protein VP6 of two Serotypes of African Horsesickness Virus. Fifth international symposium on double-stranded RNA viruses, March 1995, Jerba, Tunisia. Huismans, H. and **de Waal, P.J.** (1997) The Inner core protein VP6 of African Horsesickness Virus binds Nucleic Acids with a preference for dsRNA. International symposium on double-stranded RNA viruses, November 1997, Mexico. Huismans, H. and **de Waal, P.J.** (2003) Characterization of the nucleic acid binding activity of core protein VP6 of African horsesickness virus. 8th International Symposium on Double-Stranded RNA viruses. September 2003. Il Ciocco, Castelvecchio Pascoli, Italy.
Local Congress Participation:	Cormack, S.B., **Turnbull, P.J.** and Huismans H. (1994) Cloning and in vitro expression of two minor core proteins of African Horsesickness Virus type 3. Fourteenth Congress of the South African Genetics Society: GENETICS FOR AFRICA, June 1994, Pretoria. **Turnbull, P.J.**, Cormack, S.B. and Huismans, H. (1996) The cloning characterization and expression of a gene that encodes a possible African Horsesickness Virus helicase. Ninth Biennial Congress of the South African Society for Microbiology, July 1996, Pretoria. **De Waal, P.J.** and Huismans, H. (2000). The role of post translational modification in the nucleic acid binding property of the inner core protein VP6 of African Horsesickness virus. Seventeenth Congress of the South African Genetics Society. June 2000, Pretoria. **De Waal P.J.** and Huismans, H. (2005) Characterization of the nucleic acid binding activity of inner core protein VP6 of African horsesickness virus. XIXth South African Society of Biochemistry and Molecular Biology (SASBMB) Conference. January 2005, Stellenbosch.

CHAPTER 7:REFERENCES

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