

CHAPTER ONE

LITERATURE REVIEW

1.1. INTRODUCTION

African horsesickness (AHS) is an infectious, but non-contagious arthropod transmitted viral disease that causes high mortality in horses. In naive populations of horses, the mortality rate commonly exceeds 80% and may be as high as 95%. The disease occurs regularly in most countries in sub-Saharan Africa and is regarded as one of the major scourges of the continent. AHS was already present in South Africa in natural reservoir hosts at the time the first Dutch settlers had arrived in the Cape of Good Hope in 1652. Following the import of horses, major epidemics occurred approximately every 20 to 30 years prior to 1953. Although the virus has not maintained itself outside sub-Saharan Africa, occasional outbreaks have occurred in Egypt, Saudi-Arabia, Afghanistan, Pakistan, India as well as the relative recent outbreak in the western Mediterranean region (Coetzer & Erasmus, 1994).

The first pioneering research on AHS was carried out by Sir Arnold Theiler. He established that the causative agent was filterable through Chamberland filters capable of retaining bacteria. He concluded that the agent was a virus capable of inducing a variety of anthrax and biliary symptoms in horses. His research also indicated that immunologically distinct strains of AHS virus (AHSV) may exist, seeing as immunity acquired against one strain of virus did not always protect the horse when challenged against infection by a heterologous strain (reviewed in Coetzer & Erasmus, 1994). Theiler, together with Pitchford-Watkins, ascertained in 1903 that AHSV may be transmitted by biting insects, thereby establishing the groundwork for future research on the epidemiology, pathogenesis and aetiology of the AHS. In 1921, Theiler reported the first detailed descriptions of the clinical signs and gross lesions produced by infection with AHSV (Theiler, 1921). Alexander and co-workers were the first to succeed in propagating AHSV in chicken embryos and demonstrated that the virus became attenuated during passage in embryonated eggs (Coetzer & Erasmus, 1994). Characterisation of the AHSV structure and morphology occurred in the 1960s and early 1970s (Els & Verwoerd, 1969; Verwoerd, 1969; Oellerman *et al.*, 1970; Verwoerd *et al.*, 1970). During this time, AHSV was classified as belonging to the *Orbivirus* genus within the family *Reoviridae* (Oellerman *et al.*, 1970; Bremer, 1976).

The prevention of AHS and the spread of AHSV are of great economic importance to all affected countries, as serious losses in both the horse racing and show jumping industry could rapidly occur

if the disease is not kept under control. The export and transfer of horses from affected countries is also severely hampered due to stringent quarantine regulations, affecting the competitiveness of the horses (Coetzer & Erasmus, 1994). South Africa is considered enzootic for AHS, therefore several European and other countries prohibit the import of horses from countries like South Africa where the disease occurs or where active vaccination with live attenuated virus takes place. In order to control the disease, the following guidelines have been developed: restriction of animal movement during outbreaks of the disease, quarantine and slaughter of infected animals, control of the insect vectors (biting midges of the *Culicoides* species) and vaccination. These exercises are costly and labour intensive.

1.2. CLASSIFICATION OF THE GENUS *ORBIVIRUS*

The aetiological agent of AHS is *African horsesickness virus* (AHSV), a member of the genus *Orbivirus* in the family *Reoviridae* (Oellerman *et al.*, 1970; Bremer, 1976; Verwoerd *et al.*, 1979; Holmes, 1991; Murphy *et al.*, 1995). Other genera in the family include *Orthoreovirus*, *Rotavirus*, *Aquareovirus*, *Phytoreovirus*, *Fijivirus*, *Cypovirus*, *Coltivirus*, and *Oryzavirus* (Matthews, 1982; Francki *et al.*, 1991; Gorman, 1992; Calisher & Mertens, 1998). The main characteristic of viruses within the *Reoviridae* family is that they possess a segmented, linear, double-stranded (ds) RNA genome (generally 10-12 segments) enclosed within a double-layered capsid, and contain no lipoprotein (Matthews, 1982; Joklik, 1983; Francki *et al.*, 1991; Gorman, 1992). The virus particle or virion is icosahedral with a diameter of 60-80 nm.

AHSV is similar in morphology, physicochemical attributes and immunological properties to other orbiviruses such as *Epizootic haemorrhagic disease virus* (EHDV) of deer, *Equine encephalosis virus* (EEV) and *Bluetongue virus* (BTV), with BTV being the prototype virus of this genus (Verwoerd *et al.*, 1979; Spence *et al.*, 1984). Orbiviruses share similar physicochemical properties and morphological appearance (Borden *et al.*, 1971), although no common antigen has been found. Like the rotaviruses and orthoreoviruses, the orbiviruses are non-enveloped viruses with a diameter of 60-80 nm. Orbiviruses can be distinguished morphologically from the other genera by the presence of a characteristic diffuse outer capsid layer, surrounding the inner nucleocapsid which consists of 32 ring-shaped capsomeres arranged with icosahedral symmetry (Verwoerd *et al.*, 1969; Borden *et al.*, 1971; Murphy *et al.*, 1971; Murphy *et al.*, 1995). The genus name is derived from this characteristic ring-like structure of the capsomeres (*orbis* being Latin for ring or circle). Unlike other members of the family *Reoviridae*, orbiviruses replicate in insects and in vertebrates (Gorman & Taylor, 1985), show a greater sensitivity to lipid solvents and detergents, and virus-infectivity is lost in mild acid conditions (Murphy *et al.*, 1971; Gorman, 1978; Verwoerd *et al.*, 1979). The physicochemical properties of orbiviruses versus those of other *Reoviridae* are summarised in table 1.

Members of the genus *Orbivirus* are subdivided into serogroups based on the cross-reactivity of their antigens in complement fixation (CF), immunodiffusion and immunofluorescence tests (Gorman, 1979 & 1983; Knudson & Monath, 1990; Brown *et al.*, 1991). Orbiviruses are grouped into 19 distinct serological groups (Gorman & Taylor, 1985; Calisher & Mertens, 1998)(as summarised in Table 2). Serotypes within a serogroup are recognised by distinct reactivities in serum neutralisation tests (Gorman, 1979, 1983 & 1985) and with gene reassortment sometimes occurring between the closely related viruses (Samal *et al.*, 1987; Brown *et al.*, 1988; Cowley *et al.*, 1989). The serotypes are determined by the outer capsid proteins, VP2 and VP5, encoded by genome segments 2 and 5, respectively (Calisher & Mertens, 1998). To date, nine different serotypes of AHSV have been identified with little, if any, cross-neutralisation between them (McIntosh *et al.*, 1958; Howell *et al.*, 1962; Calisher & Mertens, 1998).

The genus *Orbivirus* includes a number of viruses, which are of agricultural importance such as the pathogenic agents of native and domestic animals. Important pathogens include AHSV, *Bluetongue virus* (BTV), *Epizootic haemorrhagic disease virus* (EHDV) of deer and *Equine encephalosis virus* (EEV) (Verwoerd *et al.*, 1979; Spence *et al.*, 1984). Other than BTV, the prototype virus of this genus, a limited amount of information is available on other orbiviruses such as AHSV. Due to similarities between BTV and AHSV many of the features of BTV can be extrapolated to AHSV. This chapter therefore mainly reviews findings with BTV, although relevant studies on AHSV are also included.

TABLE 1: *Physicochemical properties of orbivirus versus orthoreoviruses (Adopted from Verwoerd *et al.*, 1979; Gorman, 1978; Knudson & Monath, 1990).*

Property	Orbivirus	Orthoreovirus
Acid sensitivity	Sensitive	Resistant
Detergent lability	Partial instability	Stable
Temperature stability	Reasonable thermostable	Thermostable
Portal of entry	Skin via vector	Oral
Tissue tropism	Hemopoietic	Intestinal tract
Invertebrate vector	Culicoid midges, mosquitoes, phlebotomines and ticks	none

TABLE 2: Viruses of the genus *Orbivirus* by serogroups, serotypes, hosts and principle vector (Gorman & Taylor, 1985; Gorman, 1992; Holmes et al., 1995; Calisher & Mertens, 1998).

Species	Number of Serotypes	Host species	Principle vector
<i>Bluetongue virus</i> (BTV)	24	Cattle, sheep, goats, camels, ruminants	<i>Culicoides</i>
<i>African horsesickness virus</i> (AHSV)	9	Equids, camels, dogs, cattle, sheep, elephants	<i>Culicoides</i>
<i>Epizootic haemorrhagic disease</i> (EHDV)	10	Cattle, sheep, deer, etc.	<i>Culicoides</i>
	7	Equids	<i>Culicoides</i>
<i>Equine encephalosis virus</i> (EEV)	4	Unknown	<i>Culicoides</i> and
<i>Eubananguee virus</i> (EUBV)		(isolated from insects)	mosquitoes
	11	Cattle, sheep	<i>Culicoides</i> and
<i>Palyam virus</i> (PALV)			mosquitoes
	4	Birds	mosquitoes
<i>Umatilla virus</i> (UMAV)	12	Humans, rodents, sloths	Plebotomine flies
<i>Changuinola virus</i> (CGLV)			and mosquitoes
	3	Humans, rodents	Culicine mosquitoes
<i>Corriparta virus</i> (CORV)	7	Seabirds	Ticks
<i>Chenuda virus</i> (CNUV)	2	Bats	Ticks
<i>Chobar Gorge virus</i> (CGV)	3	Birds	<i>Culex</i> mosquitoes
<i>Ieri virus</i> (IERIV)	36	Seabirds, rodents,	Ticks
<i>Great island virus</i> (GIV)		humans	
	1	Humans, rodents	Culicine mosquitoes
<i>Lebombo virus</i> (LEBV)	4	Humans, camels, cattle,	Culicine mosquitoes
<i>Orungo virus</i> (ORUV)		monkeys	
	3	Marsupials	<i>Culicoides</i> and
<i>Warrego virus</i> (WARV)			mosquitoes
	3	Marsupials	<i>Culicoides</i>
<i>Wallal virus</i> (WALV)	8	Cattle, macropods	<i>Culicoides</i> and
<i>Wongorr virus</i> (WGRV)			mosquitoes
	2	Domestic animals	Ticks
<i>Wad Medani virus</i> (WMV)	1	Unknown (deer)	Ticks
<i>St Croix River virus</i> (SCRV)	8		
Unassigned viruses within species			

1.3. EPIDEMIOLOGY, TRANSMISSION AND PATHOGENESIS OF AHSV

AHS is endemic to sub-Saharan Africa (Howel, 1963; Brown & Dardiri, 1990; House *et al.*, 1992a; Mellor, 1994), although severe epizootics have occurred in North Africa, the Middle East, Asia and in certain European countries (McIntosh, 1958; Mellor, 1993, Coetzer & Erasmus, 1994). The most recent outbreaks have been reported in Spain, Portugal, Morocco and Saudi Arabia (House *et al.*, 1992a; Rodriguez *et al.*, 1992; Mellor, 1993; House *et al.*, 1994). Problems with clinical recognition, pathological diagnosis and vector traffic make AHSV a very difficult disease to control and eradicate.

AHS manifests as an acute, peracute, subacute or mild infectious disease of *Equidae* (Tomori *et al.*, 1992; Mellor, 1993). The virus is transmitted between susceptible animals, in the same manner as BTV, by biting haematophagous midges of the *Culicoides* species (Du Toit, 1944; Tomori *et al.*, 1992). *Culicoides imicola* is the major AHSV vector and is also responsible for the spread of BTV (Mellor & Boorman, 1995). An adult *Culicoides* midge becomes infective 8 days after feeding on a viraemic animal and stays infective until death. Mosquitoes of the *Aedes*, *Anopheles* and *Culex* species, as well as the brown dog tick, *Rhipicephalus sanguineus*, have also been reported to transmit the disease to susceptible animals (Lubroth, 1988 & 1992; Tomori *et al.*, 1992; Mellor, 1993). However, their role in natural transmission is not known. The distribution of AHS is limited by the requirement for susceptible vertebrate hosts and the relevant arthropod vectors and usually disappears abruptly after the first frost (Mellor, 1993).

AHSV infects horses, ponies, mules, donkeys and zebras and occasionally dogs (Brown & Dardiri, 1990; Mellor, 1994; Barnard, 1997). Horses are the most susceptible and the mortality rates commonly exceed 80%. In a naive population, AHS can kill up to 95% of horses that become infected. Mules and donkeys appear to have some natural resistance to development of severe disease and zebras are even less susceptible and may serve as inapparent carriers (Davies & Otieno, 1977; Erasmus *et al.*, 1978; Brown & Dardiri, 1990; Lubroth, 1992; Mellor, 1994; Barnard *et al.*, 1994). Various natural reservoirs exist for the disease and antibodies against AHSV have been identified in camels, goats, sheep, cattle, buffalo dogs and elephants (Tomori *et al.*, 1992; Lubroth, 1992; Barnard *et al.*, 1995). A single incident of AHSV infection in humans by neuroadapted strains of the virus (serotypes 1 and 6) has also been reported (Swanepoel *et al.*, 1992) and was confirmed by inoculation of primates with these strains (Taylor *et al.*, 1992).

Initial virus replication occurs in the lymph nodes of the animal following transfer by the bite of an infected insect. After this initial replication phase, viruses are transported throughout the body where they replicate in certain endothelial cells (Lubroth, 1992; Laegreid *et al.*, 1992). In

experimentally induced cases, the incubation period varies between five and seven days, but the more virulent the virus and the larger the infectious dose, the shorter the incubation period becomes (Burrage & Laegreid, 1994; Coetzer & Erasmus, 1994). Classically, the disease presents in four clinopathological syndromes in horses, which vary in the organs affected, the severity of the lesions, the time of onset of clinical signs and mortality rates. These 4 forms, in decreasing order of severity, are the pulmonary or peracute form, the cardio-pulmonary or mixed form, the cardiac or subacute form and mild horsesickness fever (Theiler, 1921; Erasmus, 1973; Brown & Dardiri, 1990; Mellor, 1993).

The clinical form of disease expressed has been shown to be a function of the viral virulence phenotype (Laegreid *et al.*, 1993; Burrage & Laegreid, 1994), but is not serotype dependent. According to Laegreid (1996) the immune status of horses can also influence the form of disease they develop. McIntosh (1958) stated that differentiation of disease forms appears to be related to variation in susceptibility as a result of immunity from previous infection, rather than any viral property. Erasmus (1973) reiterated this view, suggesting that the cardiac and febrile forms of AHS are usually observed in immune animals that have been infected with a heterologous virus type. However, the existence of attenuated vaccine strains of each AHSV serotype, demonstrates that viral properties can also dramatically affect the severity of the disease caused by each of the AHSV serotypes, even in serologically naive animals. Laegreid *et al.* (1993) clearly indicated that in naive horses, the clinical form of the disease is a property of the specific AHSV strain used as inoculum. Recent studies (Laegreid *et al.*, 1995; O'Hara *et al.*, 1998) of AHSV reassortants between virulent and avirulent strains have indicated that genome segments 2, 6 and 10, coding for the outer capsid proteins VP2 and VP5 and the non-structural, cell release protein NS3, appeared to have a controlling influence on the determination of AHSV virulence.

1.4. THE MOLECULAR BIOLOGY AND STRUCTURE OF ORBIVIRUSES

Profound research on the molecular biology of AHSV started in the 1980s, therefore little information is available on the structure-function relationships of genes and gene products of AHSV. However, electron microscopic and physicochemical studies indicate a close morphological and biochemical relationship to BTV, the prototype orbivirus (Oellerman *et al.*, 1970; Bremer, 1976), which has been thoroughly investigated. Much of the current knowledge and research on AHSV is based on findings from studies of BTV.

1.4.1. The virion

Orbiviruses are complex non-enveloped viruses with seven structural proteins, organised into two concentric protein shells, which displays icosahedral symmetry. Early electron microscopic studies found the diameter of most orbiviruses to be 65-70 nm (Verwoerd *et al.*, 1972; Martin & Zweerink, 1972; Verwoerd *et al.*, 1979). The icosahedral virion consists of an indistinct outer layer, which surrounds a well-defined core-particle (Els & Verwoerd, 1969; Oellerman *et al.*, 1970; Verwoerd *et al.*, 1972), which encloses ten double-stranded RNA genome segments each encoding at least one viral protein (Oellerman *et al.*, 1970; Huismans, 1979; Grubman & Lewis, 1992) (*Figure 1.1*). Three morphologically distinct particles have been identified in orbivirus-infected cells, including virions, cores and subcores. These particles have been obtained, for BTV, by stepwise removal of specific structural proteins of the virus (Huismans *et al.*, 1987; Huismans & Van Dijk, 1990). More recently, core-like and virus-like particles (CLPs & VLPs) containing no nucleic acid have been produced by the co-expression of the relevant BTV and AHSV structural proteins in expression systems (Belyaev & Roy, 1993; Maree *et al.*, 1998). In the fully infectious BTV virion, the outer capsid is composed of two major proteins (VP2 and VP5), while the core particle consists of two major [VP3(T2) and VP7(T13)] and three minor [VP1(Pol), VP4(Cap) and VP6(Hel)] components, as well as the dsRNA genome (Verwoerd *et al.*, 1972; Martin *et al.*, 1973; Els 1973; Bremer, 1976; Huismans, 1979; Huismans *et al.*, 1979).

The purified BTV virion is composed of approximately 88% protein and 12% dsRNA (Huismans & Van Dijk, 1990; Knudson & Monath, 1990; Mertens *et al.*, 1996) and contains no single-stranded adenine rich component as is found in reovirus (Martinez-Torrecuadrada, 1994), while the core contains approximately 19.5% RNA (Stuart *et al.*, 1998; Grimes *et al.*, 1998; Gouet, *et al.*, 1999). Infectivity for mammalian cells is lost if one of the proteins in the outer capsid layer is removed (Verwoerd *et al.*, 1979), although purified core particles do have infectivity, comparable to that of intact virus, for cells of the insect vector species (*Culicoides*) (Mertens *et al.*, 1996). The virions are stable in lipid solvents (Verwoerd *et al.*, 1979) and non-ionic detergents such as Triton X-100 (Huismans *et al.*, 1987; Mertens *et al.*, 1987). The virus is sensitive to acid pH, but is relatively resistant to pH changes on the alkaline side of neutrality (Stanley, 1967; Huismans *et al.*, 1987). Recent advances in cryo-electron microscopy and computer imaging, as well as the ability to synthesise BTV core-like particles (CLPs) and virus-like particles (VLPs) using baculovirus expression vectors, have greatly facilitated an understanding of the BTV virion architecture. The structure and assembly of the virus is discussed in more detail in section 2.3.

1.4.2. The viral genome

BTV was the first orbivirus found to contain a dsRNA genome (Verwoerd, 1969). Since then the segmented dsRNA nature of the genome of other orbiviruses have also been confirmed (Oellerman, 1970; Huismans *et al.*, 1979; Knudson *et al.*, 1984; Kusari and Roy, 1986). The genomic RNA contains 5' terminal Cap 1 structures (7mGpppG^(2-0m)...). The genome of AHSV is, like that of BTV, composed of 10 linear ds RNA segments that are packaged in exactly equimolar ratios, one of each segment per particle. There is no evidence for short ssRNA oligonucleotides in intact virions. The size of the ten AHSV genome segments range from 3965 to 756 bp, with a total size and total molecular weight of 19.5 kbp and $13,2 \times 10^6$ dalton, respectively (Vreede & Huismans, 1997). This is in agreement with the BTV genome of 19.2 kbp and total M_r of 13.1×10^6 (Fukusho *et al.*, 1989; Roy, 1992). The molecular weights reported for the ds RNA genomes of orbiviruses vary between 11.0×10^6 and 13×10^6 (Verwoerd *et al.*, 1972; Schnagl and Holmes, 1975; Bremer, 1976; Gorman *et al.*, 1977), which does not differ significantly from the molecular weights of the other members of the *Reoviridae* family (18.8 kbp for rotavirus-A). The RNA segments are numbered 1 to 10 in order of their migration on agarose gels or polyacrylamide gel electrophoresis (PAGE) and are grouped as three size classes, namely large, medium and small segments (designated L1-3, M4-6 and S7-10). When compared with other members of the *Reoviridae* family, the orbiviral dsRNA profiles are distinctive (Figure 1.1).

Each viral segment encodes at least one viral-specific polypeptide (Huismans, 1979; Gorman *et al.*, 1981; Grubman *et al.*, 1983; Mertens *et al.*, 1984; Pedley *et al.*, 1988; Van Dijk & Huismans, 1988), but the smallest segment encodes two related proteins from in-phase AUG initiation codons (Van Staden & Huismans, 1991; Van Staden *et al.*, 1995). The largest genome segment, S1, is 3965 bp long, while segment 10 is only 756 bp in length. All ten genome segments share some common features. These include, like other viruses belonging to the family *Reoviridae*, 5' and 3' terminally conserved hexanucleotides on each segment which are species specific (Rao *et al.*, 1983). In the AHSV species the terminal sequences are not always identical and may not be conserved in all 10 segments. In addition, the orbiviruses, contain an inverted repeat, adjacent to the conserved termini, which differs in sequence for each segment (Roy, 1989; Nel *et al.*, 1990; Moss *et al.*, 1992; Mizukoshi *et al.*, 1993). It is postulated that these termini are involved in sorting and assembly of the genome during viral replication, due to their ability to form mRNA secondary structures (Anzola *et al.*, 1987).

The coding assignment of each dsRNA segment is summarised in Figure 1.1 and the protein characteristics and functions are summarised in Table 3. The structural proteins VP1(Pol), 2, 3(T2), 4(Cap) and 7(T13) are encoded by segments 1, 2, 3, 4 and 7 respectively and VP5 and 6(Hel) by segments 6 and 9 (O'Hara *et al.*, 1993; Burroughs *et al.*, 1994). Segments 5 and 8 encode the non-

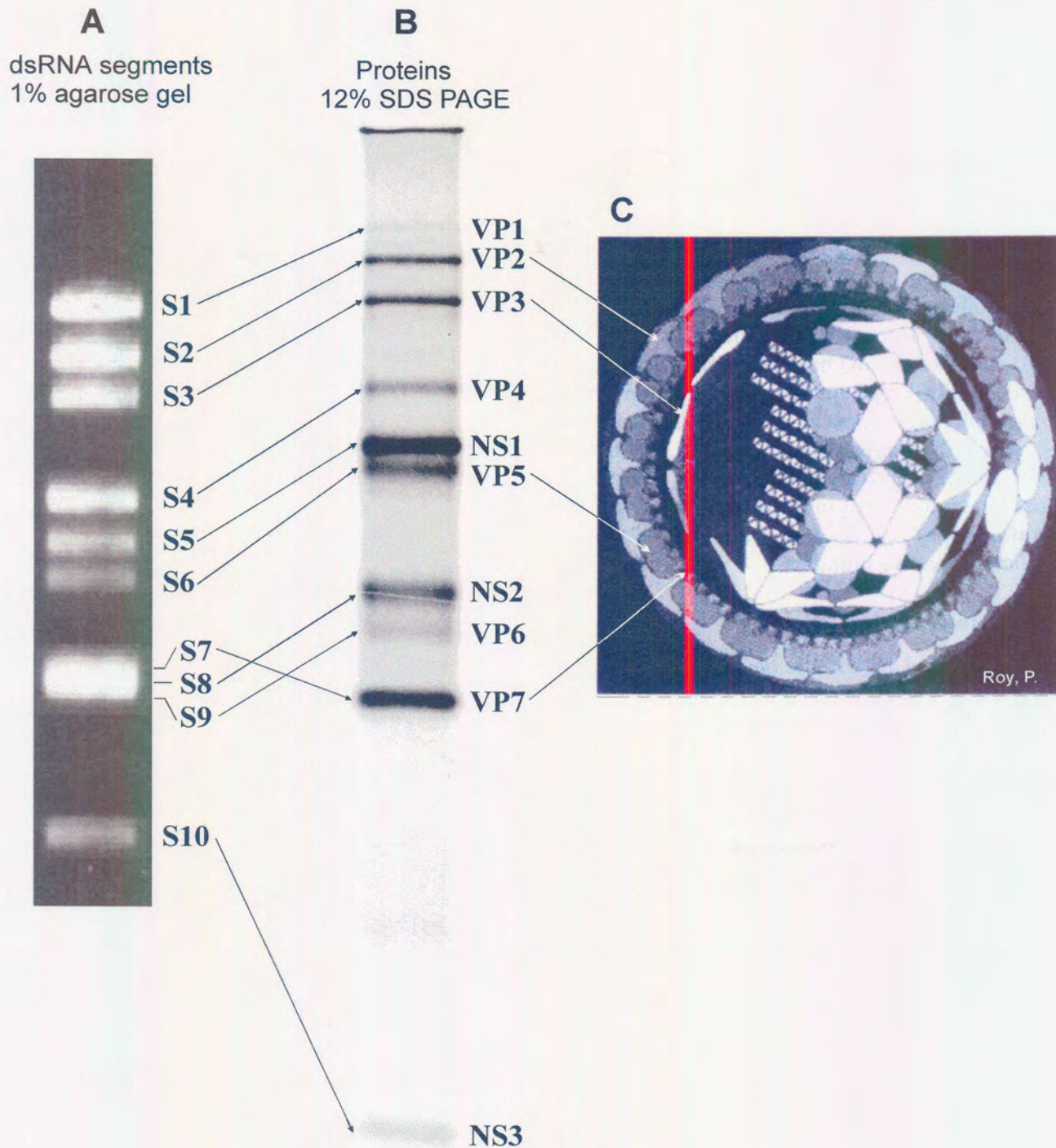


Figure 1.1: AHSV coding assignments: (a) represent gel electrophoretic separation of the dsRNA segments of AHSV-6 (adopted from Grant Napier, 1999), (b) represents the size distribution of the polypeptides, encoded by the ten dsRNA segments, on polyacrylamide gel (kindly provided by Michelle van Niekerk) and (c) a schematic diagram of the orbivirus particle (adopted from Roy, 1998). All orbiviruses share some basic morphological features with BTV, the prototype orbivirus.

TABLE 3: *AHSV genome segments and their encoded proteins. Adopted from Vreede & Huismans (1997).*

dsRNA Segm.	Sero	Segment length (bp)	Protein	Number of aa (Predicted M _r)	Function or property
1	9	3965	VP1	1305 (150 292)	Minor core structural protein; possible dsRNA-dependent ssRNA polymerase
2	3	3221	VP2	1057 (123 063)	Outer capsid structural protein; cell attachment protein; serotype specific; highly variable; protective neutralising antigen; determination of virulence
3	4	2795	VP3	905 (103 269)	Major core structural protein; subcore scaffold layer; T=2 symmetry; serogroup specific; control overall size and organisation of capsid structure
4	4	1978	VP4	642 (75 826)	Minor core structural protein; dimer; transmethylase 1 and 2; Guanylyltransferase activity (capping enzyme)
5	9	1566	VP5	505 (56 771)	Outer capsid structural protein; intra-serogroup variable gene; glycosylated; trimer
6	9	1748	NS1	548 (63 377)	Major non-structural protein; virus-specified tubular structures
7	9	1167	VP7	349 (37 916)	Major core surface structural protein; ring-like capsomeres; trimers with T=13 symmetry; serogroup specific; involved in cell entry
8	9	1166	NS2	365 (41 193)	Non-structural protein; binds ss RNA; phosphorylated; viral inclusion body (VIBs) matrix protein
9	3	1169	VP6	369 (38 464)	Minor core structural protein; possible helicase and NTPase; binds ss & ds RNA
10	9	756	NS3/ NS3A	217 (23 659) 206 (22 481)	Minor non-structural proteins; glycosylated; membrane associated; involved in virus release; determination of virulence

structural proteins NS1 and NS2, while segment 10 encodes NS3 and NS3A. These coding assignments and protein nomenclature are consistent with that of BTV.

The recent availability of nucleotide sequences of a number of different orbiviruses has made detailed comparisons of genes and gene products possible. In addition, the information to enables analysis of the structural, functional and biochemical characteristics of the proteins in an attempt to understand the molecular biology of the orbiviruses. To date, the nucleotide sequences of all ten AHSV RNA segments have been determined, although not all from the same serotype (Table 3) and their similarities with the analogous genes of BTV or other orbiviruses documented by Roy *et al.*, 1991; Van Staden and Huismans, 1991; Van Staden *et al.*, 1991; Iwata *et al.*, 1992; Mizukoshi *et al.*, 1992.

1.4.3. The viral proteins

The structural proteins are numbered VP1(Pol) to VP7(T13) in order of decreasing size based on electrophoretic migration in polyacrylamide gels, and the non-structural proteins are designated NS1, NS2 and NS3 (Huismans & Van Dijk, 1990)(*Figure 1.1*).

1.4.3.1. The outer capsid polypeptides

The outer capsid proteins, VP2 and VP5, are encoded by RNA segments 2 and 6 respectively in the case of BTV, EHDV and AHSV (Mertens *et al.*, 1984; Bremer *et al.*, 1990; Le Blois *et al.*, 1991; Iwata *et al.*, 1992; Vreede & Huismans, 1994). Of the two outer capsid proteins, VP2 is the more variable and exhibits the least conservation between serotypes and serogroups (Fukusho *et al.*, 1987; Iwata *et al.*, 1992; Vreede & Huismans, 1994). VP2 is the main determinant of serotype-specificity (Huismans & Erasmus, 1981; Kahlon *et al.*, 1983; Roy *et al.*, 1990), is the viral haemagglutinin (Cowley & Gorman, 1987; French *et al.*, 1990; Loudon *et al.*, 1991) and elicit neutralising antibodies (Mecham *et al.*, 1986; Huismans *et al.*, 1987a; Inumaru & Roy, 1987; White & Eaton, 1990; Huismans & Van Dijk, 1990; Burrage *et al.*, 1993). The protein is also associated with cell adsorption in virus infection since removal of VP2 eliminates binding of the virus to the cell (Eaton & Crameri, 1989; Van Dijk & Huismans, 1990).

VP2 seems to be a candidate for the production of a subunit vaccine. VP2 has been shown to induce serotype-specific neutralising antibodies in sheep, where BTV VP2 inoculates were protected against subsequent challenge with the corresponding virus serotype (Huismans & Erasmus, 1981; Kahlon *et al.*, 1983; Huismans *et al.*, 1987; Inumaru & Roy, 1987; Gould *et al.*, 1988; White & Eaton, 1990). In AHSV it was also found that VP2 has neutralising properties, as the neutralising epitopes of AHSV-4 were found to be located on VP2 (Burrage *et al.*, 1993). Martinez-

Torrecuadrada *et al.* (1994) have shown that antibodies raised to AHSV VP2 in rabbits neutralise a virulent strain of AHSV-4, while Stone-Marchat *et al.* (1996) have also shown that horses can be immunised using a vaccinia-based construct containing the VP2 gene of AHSV-4. The regions on VP2 that are involved in eliciting protective immune response and determination of serotype specificity have not as yet been fully identified.

In contrast to VP2, very little is known about the function of VP5 other than its close association with the core particle. The protein is less variable than VP2 (Gould & Pritchard, 1988; Wade-Evans *et al.*, 1988; Oldfield *et al.*, 1991; Iwata *et al.*, 1991; Du Plessis & Nel, 1997), suggesting a high degree of restraint on the structural variability of VP5. Although the protein is located in the outer capsid, it is mostly unexposed (Hewat *et al.*, 1992; Iwata *et al.*, 1992) and it does not appear to have any distinct neutralising ability on its own (Mertens *et al.*, 1989). Antisera raised against baculovirus expressed VP5 protein do not demonstrate any neutralising activity *in vitro* (Marshall & Roy, 1990), however there are indications that VP5, in the combination with VP2, does contribute to the induction of neutralising antibodies in BTV infection (Mertens *et al.*, 1989; Roy *et al.*, 1990). It is postulated that although VP5 does not elicit neutralising antibodies on its own, it may enhance the immune response to VP2 by affecting the conformation of VP2, and hence the serological properties of the protein (Roy *et al.*, 1996). It is thought that these proteins stabilise each other in the virion outer capsid during virus morphogenesis (Liu *et al.*, 1992).

1.4.3.2 The core polypeptides

In virus-infected cells, BT or AHS virions are converted to core particles with the removal of the outer capsid proteins, VP2 and VP5 (Verwoerd *et al.*, 1972; Els 1973). The diameter of the core particles is in the order of 73 nm (Mertens *et al.*, 1987; Hewat *et al.*, 1992; Grimes *et al.*, 1998). The VP3(T2) and VP7(T13) proteins are the two major components of the core (Verwoerd & Huismans, 1972, Huismans *et al.*, 1987), VP7(T13) being the more abundant. These two major core proteins are highly conserved between orbiviruses (Iwata *et al.*, 1992; Roy *et al.*, 1991; Bremer *et al.*, 1994) and are associated in a highly ordered, three-dimensional manner in the core, resulting in icosahedral symmetry (Grimes *et al.*, 1998).

VP7(T13), the major serogroup-specific antigen, is located on the surface of the core (Huismans & Erasmus, 1981; Oldfield *et al.*, 1990; Chuma *et al.*, 1992). This major component of the core is highly conserved among serotypes (Bremer *et al.*, 1990; Roy *et al.*, 1991; Iwata *et al.*, 1992). It is an extremely hydrophobic protein, and has been demonstrated to form trimers (Kowalik *et al.*, 1990; Roy, 1992). This hydrophobicity is particularly evident in AHSV VP7(T13) where it self-assembles to form disc shaped, hexagonal crystalline structures of various sizes (Chuma *et al.*, 1992; Burroughs *et al.*, 1994). Such crystals have so far never been observed for other orbiviruses. AHSV-4

VP7(T13) has an estimated M_r of 38.0 kDa (Roy *et al.*, 1991). The VP7(T13) protein of different orbiviruses contains a conserved Arg-Gly-Asp (RGD) tripeptide motif, which has been suggested as a possible determinant for attachment of VP7(T13) to a cell surface receptor. This tripeptide is present on almost all known orbivirus VP7(T13) proteins and is situated in an exposed position of the viral core (Eaton *et al.*, 1991; Grimes *et al.*, 1995).

The third largest RNA segment encodes VP3(T2), which is, like VP7(T13), also hydrophobic in nature and contains group specific antigens (Iwata *et al.*, 1992; Inummaru *et al.*, 1987). Sequence data previously obtained for the VP3(T2) gene and deduced protein of BTV, AHSV and EHDV, indicates that of the four major capsid proteins, VP3(T2) is the most conserved (Iwata *et al.*, 1992). VP3(T2) plays an important role in the structural integrity of the virus core and has been proposed to form the protein scaffold on which the capsomers, of which VP7(T13) are the main component, are arranged (Huismans *et al.*, 1987a). AHSV-4 VP3(T2) is composed of 905 amino acids with a predicted size of 103.27 kDa (Iwata *et al.*, 1992) and the amino acid content and size is similar to the BTV and EHDV VP3(T2) proteins (Le Blois *et al.*, 1991). VP3(T2) has also been shown to have an affinity for ssRNA (Loudon & Roy, 1992).

Inside the subcore, VP3(T2) acts as a framework for interaction with the minor proteins VP1(Pol), VP4(Cap) and VP6(Hel). The core particles are transcriptionally active and are associated with an RNA-dependent RNA polymerase activity (Van Dijk & Huismans, 1980; Verwoerd & Huismans, 1972). This enables the virus to synthesise mRNA from the virion dsRNA templates. Based on its size ($M_r = 150$ kDa), location and molar ratio (estimated at 10 molecules per virion) (Stuart *et al.*, 1998), VP1(Pol) is the prime candidate for the RNA polymerase. BTV VP1(Pol) has also shown homology with a vaccinia virus DNA-dependent RNA polymerase subunit (Roy *et al.*, 1988; Urakawa *et al.*, 1989; Vreede & Huismans, 1998), as well as several other prokaryotic and eukaryotic RNA polymerases (Kowalik *et al.*, 1990; Kowalik & Li 1989). VP4(Cap) is thought to be responsible for the guanylyl transferase activity based on results indicating that VP4(Cap) of BTV binds GTP. VP4(Cap) has therefore been associated with mRNA 5' capping and methylation during transcription. (Le Blois *et al.*, 1992; Roy, 1992; Mizokoshi *et al.*, 1993; Ramadevi *et al.*, 1998). However, VP1(Pol) and VP4(Cap) are thought to have a co-operative enzymatic function in RNA transcription and/or RNA replication (Huismans & Van Dijk, 1990). It has recently been shown that BTV VP4(Cap) has nucleoside triphosphate phosphohydrolase activity with specific substrate preference, viz. GTP>ATP>UTP>CTP (Ramadevi & Roy, 1998) and has capping activity (Ramadevi *et al.*, 1998). The VP6(Hel) protein of BTV, is rich in charged amino acids and is reported to possess single and double stranded RNA binding properties (Roy *et al.*, 1990; Hayama & Li, 1994) and sequence analysis has revealed a motive common to several helicases (Roy, 1992; Turnbull *et al.*, 1996). Such an activity may be involved in unwinding the dsRNA prior to transcription and

replication. VP6(Hel) may also have a role in the encapsidation of the RNA (Roy, 1992). All three minor proteins are closely associated with the ten dsRNA segments.

1.4.3.3. The non-structural proteins

At least four non-structural viral proteins are synthesised in orbivirus-infected cells. NS1 and NS2 are both synthesised abundantly in orbivirus-infected cells, while only small amounts of NS3 and NS3a are produced (Bremer *et al.*, 1976; Huismans, 1979; Mertens *et al.*, 1984; Van Dijk & Huismans, 1988; Mecham & Dean, 1988; Whistler & Swanepoel, 1990; Grubman & Lewis, 1992; O'Hara *et al.*, 1993). Little is known about the roles of the non-structural proteins in orbivirus replication cycle, although it is believed that these proteins are involved in the process of viral morphogenesis, leading to viral assembly and release. The two major non-structural proteins (NS1 and NS2) coincide with the appearance of two virus specific structures, namely tubules and virus inclusion bodies (VIBs), which characterise the cytoplasm of cells infected with orbiviruses (Lecatsas, 1968; Cromack *et al.*, 1971; Murphy *et al.*, 1971; Huismans, 1979).

The synthesis of NS2 in orbivirus-infected cells coincides with the synthesis of granular viral inclusion bodies (Roy *et al.*, 1990; Roy, 1992). VIBs have both granular and fibrillar characteristics and are found throughout the cell, but predominantly in proximity to the nucleus (Eaton *et al.*, 1990). NS2 is the major component of the VIBs observed in BTV-infected cells (Eaton *et al.*, 1987; Thomas *et al.*, 1990; Brookes *et al.*, 1993). In addition to NS2, these VIBs also contain ssRNA, dsRNA, NS1, structural proteins as well as both complete and incomplete viral particles (Eaton & Hyatt, 1988; Eaton *et al.*, 1990; Hyatt *et al.*, 1992; Brookes *et al.*, 1993). These observations have led to the recognition of these inclusion bodies as the sites in which the viral assembly process occurs. The NS2 protein of various orbiviruses has been reported to bind ssRNA (Huismans & Basson, 1983; Huismans *et al.*, 1987; Thomas *et al.*, 1990; Theron *et al.*, 1994; Uitenweerde *et al.*, 1995) and forms a complex in the presence of virus mRNA. It has been suggested that NS2 may play a role in the selection and condensation of the 10 viral ssRNA species into precursor subviral particles, prior to dsRNA synthesis. A distinctive feature of NS2 is that it is the only orbivirus-encoded phosphoprotein (Huismans & Basson, 1983; Huismans *et al.*, 1987; Devaney *et al.*, 1988; Theron *et al.*, 1994), but its significance in NS2 activity is unknown. The phosphorylation of NS2 seems to down regulate its ssRNA binding ability (Theron *et al.*, 1994).

The two smallest and closely related non-structural proteins NS3 and NS3A are synthesised in low abundance in orbivirus-infected cells (French *et al.*, 1989; Wu *et al.*, 1992; Van Staden *et al.*, 1995). NS3 and NS3A proteins are co-linear products encoded by the genome segment 10 and the two proteins are translated from different in-frame initiation codons (Mertens *et al.*, 1984; Lee & Roy, 1987; Van Dijk & Huismans, 1988; French *et al.*, 1989; Van Staden & Huismans, 1991; Wu *et al.*,

1992). Thus, it is possible that NS3A is essentially a truncated form of NS3. Both NS3 and NS3A are glycosylated, contain two conserved hydrophobic regions implicated to act as transmembrane regions and are involved in virus release from infected cells (Wu *et al.*, 1992; Hyatt *et al.*, 1991; Hyatt *et al.*, 1993; Van Staden *et al.*, 1995).

NS1:

Virus tubules are present in large numbers predominantly in peri- or juxtannuclear locations. These morphological structures are believed to be attached to the intermediate filament components of the cytoskeleton of the cell (Eaton *et al.*, 1987; Eaton *et al.*, 1988; Eaton & Hyatt, 1989) and are presumed to be involved in some way in the virus replication or transportation process. The unique virus-specified tubular structures observed in the cytoplasm of orbivirus-infected cells are composed of NS1 subunits (Murphy *et al.*, 1971; Huismans & Els, 1979, Urakawa & Roy 1988; Nel & Huismans., 1991). NS1 is encoded by RNA segment 5 (Urakawa & Roy, 1988; Nel & Huismans, 1991). This 64K non-structural protein is expressed more abundant than any other BTV protein in infected cells, comprising about 25% of the virus-specified protein complement (Huismans & Els, 1979). In a number of different orbiviruses investigated, it has been found that the ten different mRNA species are not synthesised in equimolar amounts. Instead, the dsRNA genome segment encoding NS1 is transcribed at a higher rate than the other genome segments (Huismans & Verwoerd, 1973; Venter *et al.*, 1991). During virus infection the NS1 protein is synthesised in large excess over the other viral proteins, but very little soluble NS1 is observed in the cytoplasm. The NS1 polymerises very rapidly into high molecular weight tubular structures (Huismans, 1979; Huismans & Bremer, 1979; Huismans & Els, 1979).

The NS1 proteins are highly ordered structures, most likely due to the large number of conserved cysteine residues and the major hydrophilic and hydrophobic regions scattered throughout the protein (Fukusho *et al.*, 1989, Nel *et al.*, 1990). Monastyrskaya *et al.* (1994) have revealed several conserved elements in the protein which are necessary for tubule formation, with cysteine residues at positions 337 and 240 and the conserved carboxyl- and amino-terminals shown to be of particular importance. The assembly of NS1 in tubular structures and the function of the tubules in orbivirus replication are unknown. Some virions and inner core particles have been observed in association with tubules in the cytoplasm of BTV-infected cells (Eaton *et al.*, 1988). Brookes *et al.* (1993) have reported that BTV NS1 is present within distinct areas of the VIBs and that these areas correlated with the presence of virus particles of different size dimensions (i.e. particles in different stages of morphogenesis), indicating that NS1 is involved in the early stages of morphogenesis. Another hypothesis is that NS1 may be involved as molecular chaperons to prevent the core particles from assembling before the correct minor core proteins [VP1(Pol), VP4(Cap) and VP6(Hel)] and/or genome have been incorporated (Hewat *et al.*, 1992).

1.5. ORBIVIRUS MORPHOGENESIS AND VIRAL REPLICATION

Following infection of a susceptible animal, BTV binds to a receptor in the cell membrane and the virus is believed to enter the cells by receptor-mediated endocytosis. No information is available on the nature of the cell receptor to which BTV or AHSV binds. The adsorption of the viruses to mammalian cells is mediated by VP2. Huismans *et al.* (1983) demonstrated that particles of BTV lacking VP2, but containing the other outer coat protein VP5, were unable to adsorb to cells in suspension. Although core particles of BTV bind to mammalian cells, they show very low infectivity, perhaps because these cells cannot correctly internalise them. The outer capsid proteins are therefore important for mammalian cell infection. Cores however, are highly infectious for cells of the insect vector *Culicoides variipennis*, which suggests infection of these cells, is via a different mechanism (Mertens *et al.*, 1996).

After endocytosis, the clathrin-coated vesicles are transported to the vicinity of the nucleus where they fuse to form endosomes (Eaton *et al.*, 1990), and BTV probably enters the cytoplasm by penetration of the endosomal membrane. Shortly after infection, BTV virions are converted to core particles, which have an active RNA transcriptase, and then to subcore particles, containing VP3(T2) and the three minor core proteins (Huismans *et al.* 1987). The conversion of BTV to core particles by removal of both outer capsid proteins VP2 and VP5 may occur in the endosome, which has an acid pH, or may be completed in the cytoplasm after partial uncoating in the endosome (Huismans *et al.*, 1987; Eaton *et al.*, 1990). Removal of both capsid proteins is required to activate the virion transcriptase and mRNA synthesis (Van Dijk & Huismans, 1980), by a currently unknown mechanism. The most likely explanation is that this modification allows free access of the NTPs to the genome and the unimpaired extrusion of the newly synthesised mRNA (Huismans & Van Dijk, 1990).

Because the dsRNA would trigger host defence mechanisms if released into the cytoplasm, dsRNA viruses retain their genomes within a protein core that contains active dsRNA-dependent ssRNA polymerases (transcriptases) and for most eukaryotic dsRNA viruses, capping enzymes (Furuichi *et al.*, 1975; Gouet *et al.*, 1999). The core particle is therefore vital for function, acting as an enzyme complex that produces full-length, capped mRNA copies simultaneously from each of the genome segments it contains.

Following release from endosomes, the transcriptionally active core particles in the cytoplasm rapidly become associated with a matrix and transcription of the viral RNA occurs. This matrix is similar in structure, but less dense, than that found in mature VIBs (Hyatt *et al.*, 1991; Fu *et al.*, 1995). Presumably the viral mRNA transcripts which are transported to the cytoplasm, and the

proteins which are translated from the viral mRNA, condense around the parental cores to form VIBs (Brookes *et al.*, 1993). The transcripts, function not only to encode proteins, but also as a template for production of minus strands to form the dsRNA genome segments encapsidated in the progeny virions (Furuichi *et al.*, 1976). The interaction of NS2 with ssRNA, the predominant form of RNA within VIBs, probably leads to the formation of VIBs. In addition to this selection and condensation of virus-specific mRNA, among cellular RNAs, there has to be a segment specific recognition, which allows the encapsidation of only one copy of each segment into the progeny virion (Hyatt *et al.*, 1988; Eaton *et al.*, 1990). It is not yet clear which proteins are involved in this process, and what the exact mechanisms of recognition and selection are. Structural proteins are translated on neighbouring ribosomes and condense predominantly at the VIB periphery to form subcores and cores. Once these structures have been formed, the ssRNA is copied to form dsRNA.

Proteins VP3(T2), VP5, VP7(T13), VP6(Hel) and NS1 have also been identified in association with VIBs. Core-like and subcore-like particles are observed within VIBs, although double-shelled virions are only detected at the periphery of VIBs (Huisman *et al.*, 1987; Gould *et al.*, 1988; Hyatt & Eaton, 1988; Brookes *et al.*, 1993; Fu *et al.*, 1999). The NS1 protein is not uniformly distributed throughout the inclusion bodies, but is present within distinct areas which correlate with the presence of core and virus particles (Eaton *et al.*, 1988). This led Hyatt *et al.* (1992) to speculate that the addition of NS1 to the core particles may prevent transcription of virion RNA by progeny particles lacking their complement outer capsid proteins, hindering the release of immature virions. The addition of NS1, alternatively, may facilitate the efficient addition of VP2 and/or VP5 to the core particles at the periphery of the VIB. Since the outer capsid protein VP2 is only present on structurally complete viruses at the periphery of VIBs, it could be reasoned that virus particles are assembled at the edge of VIBs and are released into the cytoplasm upon addition of VP2 (Gould *et al.*, 1988; Brookes *et al.*, 1993).

Immediately following the addition of these outer coat proteins, the virus particles appear to associate with the intermediate filaments of the cells' cytoskeleton. VIBs, virus-specified tubules, and virus particles are associated with the cytoskeleton in BTV-infected cells (Eaton *et al.*, 1987). The viruses can be present singly, in groups, or in linear arrays (Eaton *et al.*, 1987). Both outer coat proteins VP2 and VP5 are required for stable virus-cytoskeleton interaction (Hyatt *et al.*, 1993). It is unlikely that the progeny viral particles of 65 nm in diameter can diffuse through the cytoplasm of a viable cell from the site of synthesis to the cell surface. The intermediate filaments enclosing the viral particles may provide a route whereby the progeny viruses are transported through the cell to the regions of the plasma or intracellular membranes containing NS3. These viruses are then released from the infected cells in a number of different ways. The virus might be spontaneously released as a result of cell death and subsequent cell lysis. Viruses are also released both as

enveloped particles by budding through the plasma membrane, and as non-enveloped particles by extrusion through the membrane (Hyatt *et al.*, 1989). It may also be postulated that BTV bound to the cytoskeleton filaments could facilitate interaction with NS3/NS3A manifested in smooth-surfaced vesicles, which may be transported to the plasma membrane. Here they may fuse with the membrane and facilitate the localised release of viruses from the cell (Hyatt *et al.*, 1993). The release of virions is accompanied by the removal or conformational change of VP2, resulted in the increased accessibility of VP7(T13) (Hyatt *et al.*, 1992). The following superinfection of BTV-infected cells by progeny virions effectively increases the multiplicity of infection and enhances the kinetics of BTV replication (Hyatt *et al.*, 1989).

1.6. ORBIVIRUS ASSEMBLY

BTV, the prototype orbivirus, has been the subject of extensive molecular and genetic studies, to resolve the mechanisms involve in the intracellular assembly process and the stoichiometry of the virus components in the morphogenic pathways of virions. Until recently, the mechanism by which an architecturally complex virus such as BTV is synthesised, the temporal order of virion construction and the specific interaction of the protein components to produce the icosahedral core particle were not known. Significant advances in orbivirus research have been made in recent years using gene manipulation techniques and by employing baculovirus expression system combined with 3D image reconstruction of virion particles and cores, and the X-ray crystallographic structure of certain proteins. Considerable insight has been gained into the intricate organisation and topography of the individual viral components.

1.6.1. Three-dimensional structure of orbivirus cores and core-like particles

The 3D structure of BTV core particles has been determined to a resolution of 3 nm using cryo-electron microscopy and computer image reconstruction (Hewat *et al.*, 1992; Prasad *et al.*, 1992). The particles are 69 nm (690 Å) in diameter and exhibit an icosahedral symmetry. The main morphological feature of the core particle is the presence of 32 distinct morphological units or capsomeres with a circular configuration which are organised with a triangulation number of 13 (Els & Verwoerd, 1969; Prasad *et al.*, 1992; Grimes *et al.*, 1997). The core structure is divided into two concentric layers of protein enclosing the inner core, containing the genomic dsRNA and minor proteins [VP1(Pol), VP4(Cap) and VP6(Hel)]. The surface layer of the core comprises rings or clusters of VP7(T13) trimers (Basak *et al.*, 1992; Grimes *et al.*, 1995) providing 260 prominent knob-like protrusions (780 VP7(T13) molecules; M_r 38K) organised into pentameric and hexameric units with channels in between. The 780 molecules of VP7(T13) are the main components of the virion

and are located in 260 trimeric units as a middle layer bridging the outer capsid and the inner subcore. These trimers are the basis from which the complex surface lattice of the viral core is built (Grimes *et al.*, 1995; Stuart *et al.*, 1998) and appear as triangular columns with distinct inner and outer domains, forming the attachment sites for the two surface proteins. The trimers extend outwards to a radius of 34.5 nm, from an inner radius of 30 nm. The VP7(T13) clusters are arranged so that there are 132 channels into the core interior, involving all the threefold axes and with dimensions approximately 70 Å deep and 80 Å wide at the surface. Some of the channels penetrate through the inner layer and are probably the pathways for metabolites to reach the sites of viral mRNA transcription and for the transportation of nascent mRNA molecules out of the cores to the cell cytoplasm to initiate viral protein synthesis. (French & Roy, 1990; French *et al.*, 1990; Le Blois *et al.*, 1991; Loudon and Roy, 1991; Hewat *et al.*, 1992; Liu *et al.*, 1992; Prasad *et al.*, 1992; Roy 1996).

The underlying scaffold (*Figure 1.2*) upon which the clusters of VP7(T13) trimers are arranged, consists of the second major core protein, VP3(T2) (M_r 100K). This inner layer or subcore particles has a radius of between 21.5 and 28 nm, is composed of 120 copies of VP3(T2) arranged as 12 closely bonded decamers, and has a relative smooth outer surface for attachment of the VP7(T13) trimers (Prasad *et al.*, 1992; Stuart, *et al.*, 1998). It appears that the VP3(T2) molecules form the building blocks for the icosahedral structure. The 120 molecules of VP3(T2) per virion are organised with a triangulation number of $T = 2$ (Burroughs *et al.*, 1995; Grimes *et al.*, 1998) forming two sets (A and B) of 60 subunits each (Grimes *et al.*, 1998). Thus, the ratio of VP7(T13) to VP3(T2) has been estimated to be 13:2 (Burroughs *et al.*, 1995) which is not in agreement with the earlier estimation of 13:1 (Hewat *et al.*, 1992). Until recently it was not understood, how 120 subunits of VP3(T2) molecules of dsRNA viruses, build an icosahedral shell, an ability that is inexplicable within the current conceptual framework (Caspar & Klug, 1962). The VP3(T2) subcore can retain its structure if the outer VP7(T13) core layer is lost (Huismans *et al.*, 1987; Loudon & Roy, 1991). Inside the subcore, VP3(T2) acts as a framework for interaction with the minor proteins VP1(Pol), VP4(Cap) and VP6(Hel). It is currently not known how the other viral components are organised with respect to each other, or to VP3(T2) and VP7(T13).

The images of baculovirus-synthesised CLPs revealed a similar icosahedral configuration ($T = 13$ for the surface layer) and an identical diameter (approximately 70 nm) to that of BTV cores. Some of the synthetic particles lacked the full complement of VP7(T13) and, as a consequence, the shapes of VP7(T13) trimers were more clear (Hewat *et al.*, 1992). The trimers have tripod-like shapes (8.0 nm in height) and each consists of an upper (outermost) and lower (innermost) domain. The shapes and structures of VP7(T13) trimers have recently been confirmed by X-ray crystallographic data. It appears that the VP3(T2) molecules are organised into unique plate-like structures and that

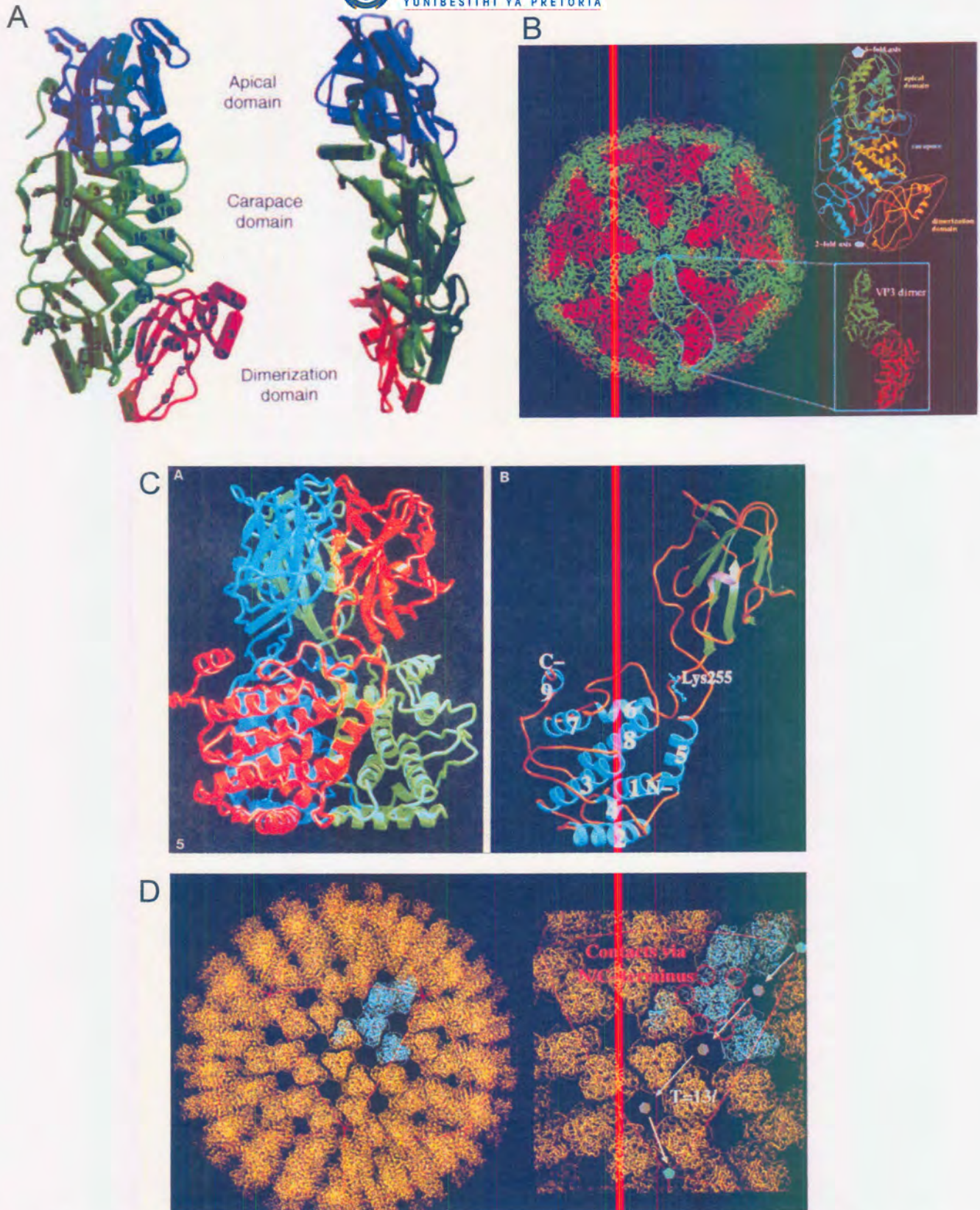


Figure 1.2: (A) A representation of the secondary structural elements of the BTV VP3(T2)B molecule (standard view left and orthogonal view right). The apical domain (residues 297-588) is colored blue, the carapace (residues 7-297, 588-698 and 855-901) is green and the dimerisation domain (residues 698-855) is red (adopted from Grimes *et al.*, 1998). (B) The architecture of the VP3 layer of the BTV core particle. The A and B subunits are shown in green and red respectively. The VP3(T2) is arranged as decamers (adopted from Stuart *et al.*, 1998). (C) A trimer and a monomer of orbivirus VP7 (T13). The protein from BTV is shown, although AHSV VP7 was found to be virtually indistinguishable (adopted from Basak *et al.*, 1997). (D) The architecture of the VP7 (T13) layer of BTV. The trimers are shown in yellow. The icosahedral 5-fold axes are joined by red lines. The right hand panel shows a close-up: The organisation of the trimers over the surface of the core is clarified in blue (adopted from Stuart *et al.*, 1998).

decamers of VP3(T2) form the building blocks for the icosahedral structure. The subcore-like particle is noticeably thicker around the fivefold axes. The disk-shaped configuration of VP3(T2) is clearly visible in the case of the CLP, due to the fact CLPs appear to be empty, so that the contrast between the inner shell and the interior is greater than that in virus-derived cores (Hewat *et al.*, 1992). In isolation, VP3(T2) will self-assemble to form particles (Moss & Nuttal, 1994; Grimes *et al.*, 1998), it binds RNA and the smallest viral particles containing RNA are VP3(T2) subcores (Loudon & Roy, 1991; Fu *et al.*, 1999). VP3(T2) seems to play a fundamental role in the early stages of BTV core assembly.

The ability of VP7(T13) trimers to interact with VP3(T2) subcores and form CLPs has been used to identify the regions of VP7(T13) necessary for the formation of particles. A number of site specific mutants and deletion or extension mutants of VP7(T13) were generated and expressed in the presence of VP3(T2). The data indicated that the intact carboxyl terminus of the BTV VP7(T13) molecule is critical for CLP formation and that the amino terminus can be modified by the addition of foreign sequences without compromising the ability to form the CLP structures. However, the CLPs that were formed were physically less stable than those formed with unmodified VP7(T13) and VP3(T2). A hydropathic plot of VP7(T13) indicates the existence of hydrophobic domains involving the last 50 amino acid residues of VP7(T13). Such domains may be involved in intra- and intermolecular interactions important for the function of the protein in particle formation (Belyaev & Roy, 1992; Le Blois & Roy, 1993).

1.6.2. Three-dimensional atomic structure of core particles

The BTV core represent the puzzle of how a large complex containing mismatched symmetry, can assemble. Results obtained by Grimes *et al.* (1998) and Gouet *et al.* (1999) from the X-ray crystallographic structure of *Bluetongue virus* core particles greatly facilitated an understanding on the assembly of bluetongue and other orbiviruses.

The outer layer of the core is built up of 13 icosahedrally independent copies of the VP7(T13) protein, in the form of four trimers in general positions and one situated with its molecular threefold axis aligned with the icosahedral threefold axis. These trimers are arranged on a pseudo-hexagonal $T = 13$ lattice and are all very similar to each other and are almost indistinguishable from the structure observed in monoclinic crystals of the recombinant VP7(T13) protein. Thus the $T = 13$ lattice follows the precept of classical quasi-equivalence (Caspar & Klug, 1962; Johnson & Speir, 1997) to an extraordinary degree. The quasi-equivalent trimers build up the core surface layer, such that they are arranged in pairs related by almost exact, local twofold symmetry axes, which are perpendicular to the surface of the virus particle. The contact regions between the different

VP7(T13) trimers are located in a thin band within the lower α -helical domain and are essentially 1-dimensional in nature. This region is formed in large part by a short hydrophobic α -helix, which seems to act as a hinge, about which the trimers are able to roll, as they pack down onto the surface of the inner core of VP3(T2). This rolling enables the same modes of interaction to be used between the different trimers, with minimal distortion (Grimes *et al.*, 1998; Stuart *et al.*, 1998).

VP3(T2) is highly unusual being organised into a structure that resembles a triangular wedge (13 Å by 75 Å) (*Figure 1.2*). The 901 amino acids of VP3(T2) are organised into a unique plate-like structure of three domains: an apical domain that sits nearest to the icosahedral 5-fold axis, a carapace domain which forms a rigid plate, and a dimerization domain that is involved in forming the quasi-2-fold interaction between the different molecules of VP3(T2). None of these domains resembles any other protein structure yet seen. The icosahedral building block of the subcore-like particle contains two molecules of VP3(T2) which perform different structural roles. The 60 VP3(T2)A subunits define the size of the subcore by forming a continuous scaffold of pairs, which span icosahedral 2-fold axes and link adjacent 5-fold axes. This scaffold is sealed off by the 60 VP3(T2)B subunits, which cluster at the icosahedral 3-fold axes to form triangular plugs. A tightly packed shell is achieved by distortion of these VP3(T2) molecular building blocks. The conformational changes occur primarily at two places in the VP3(T2) molecules: the dimerisation domain, which is intimately involved in forming the quasi-2-fold interface between molecules A and B, and between the carapace and apical domains. Ten of the VP3(T2) subunits are arranged around each five-fold axis, in the form of a decamer. The subtle rotation of the dimerisation domains is such that there is an almost exact local 2-fold relationship between dimerisation domains of the A and B molecules in adjacent decamers, so that an A molecule clips onto a B molecule in the neighbouring decamer and vice versa (Grimes *et al.*, 1998; Stuart *et al.*, 1998). This fragile internal scaffold carries the information that defines the size of the virus. This minimum possible conformational distortion, achieved by geometrical quasi-equivalent contacts, explains how 120 triangular subunits of VP3(T2) can build an icosahedral shell.

The VP7(T13) and VP3(T2) layers interact through flattish, predominantly hydrophobic surfaces (*Figure 1.2*). As a direct consequence of the symmetry mismatch between the two layers of the core there are 13 different sets of contacts between the 13 copies of VP7(T13) packed onto the 2 copies of VP3(T2) (*Figure 1.2*). Both VP3(T2)A and VP3(T2)B molecules make completely different yet extensive contacts with the various VP7(T13) molecules, whose structures are virtually identical to one another. The VP7(T13) subunits form themselves into trimers, which may then assemble into flat sheets of hexameric rings. The outer core layer is being completed by the crystallisation of 260 preformed essentially identical VP7(T13) trimers onto a fragile subcore made up of 120 molecules of VP3(T2). The driving force for this assembly of the outer core layer would be the interaction of

the relatively flat surfaces of each trimer with the underlying VP3(T2) layer, with the relative orientation of adjacent trimers of VP7(T13) determined by their side-to-side interaction with each other (Grimes *et al.*, 1998; Stuart *et al.*, 1998). This two dimensional crystallisation model avoids the need for conformational switching in the VP7(T13) layer, a mechanism used by less complex viruses for controlled capsid assembly (Liddington *et al.*, 1991; Johnson & Speir, 1997).

1.6.3. X-ray crystallographic structure of VP7(T13) trimers

The atomic structure of both AHSV and BTV VP7(T13) has recently been resolved to a 2.3 Å resolution (Grimes *et al.*, 1995; Basak *et al.*, 1996) and was found to be very similar. VP7(T13) has a unique molecular architecture not seen previously among viral proteins. The X-ray crystallographic structure of BTV VP7(T13) (*Figure 1.2*) has revealed that the three molecules of VP7(T13) interact extensively to form tightly assembled trimers with relative flat bases which can be oriented unambiguously onto the VP3(T2) subcores (Grimes *et al.*, 1995; Stuart *et al.*, 1998). The three-fold axis of the trimer (85 Å) is perpendicular to the core surface and the broader base (65 Å) of the trimer contacts the internal network of VP3(T2) of the subcore. The base of BTV VP7(T13), which is in contact with VP3(T2) in the virus, is more hydrophobic, while its upper part, which is in contact with the VP2 or VP5 outer capsid in the virus, is slightly more hydrophilic. These changes may reflect complementary changes on the other proteins involved in these interactions (Basak *et al.*, 1996).

In both BTV and AHSV each VP7(T13) subunit of the trimeric structure consists of two distinct domains, the upper or outer and the lower or inner domain, and are twisted such that the top domain of one monomer rests upon the lower domain of the related subunit (*Figure 1.2*). The relative disposition of these domains is such that they wrap around the molecular three-fold axis in a right-handed sense. The crystal structure of BTV VP7(T13) (Grimes *et al.*, 1995) exhibits an anti-parallel β -sandwich structure for the upper domain, resembling the jelly roll motif of the influenza virus haemagglutinin (Wilson *et al.*, 1981) and many other viral capsid proteins. The smaller upper domain of VP7(T13) forms the outer surface of the core and contains the central one third of the polypeptide chain of the molecule. The lower domain of VP7(T13), which contains both the N-terminus and the C-terminus of the molecule, is composed entirely of α -helices (nine in total) and long extended loops. The α -helical bottom domain is not commonly observed among viral capsid proteins. The α -helical and β -sheet domains of VP7(T13) fold independently involving specific amino acid interactions within these domains. The lower part of the bottom domain is composed of the N-terminal helices 1 to 5. The crystallographic data have implicated the lower domain, especially the flat hydrophobic area on helix 2 and the adjoining loops at the base of the VP7(T13) trimers are involved in the interaction with the underlying VP3(T2) molecules (120 VP3(T2) molecules,

arranged as dimers)(Burroughs *et al.*, 1995; Monastyrskaya *et al.*, 1997; Basak *et al.*, 1996; Grimes *et al.*, 1998). The trimers also form side-to-side interactions with adjacent VP7(T13) trimers. Each VP7(T13) has a short C-terminal arm, which involve helix 9, and may tie trimers together during capsid formation. The deletion of five amino acids at the C-terminus of VP7(T13) abolishes CLP formation, presumably due to lack of trimer-trimer interactions (Le Blois & Roy, 1993; Belyaev & Roy, 1992).

The characteristics of the molecular surface of BTV and AHSV VP7(T13) suggest why AHSV VP7(T13) is less soluble than BTV VP7(T13) and indicate the possibility of attachment to the cell via an Arg-Gly-Asp (RGD) motif in the top domain of VP7(T13), to a cellular integrin. The top domain of AHSV VP7(T13), like BTV, involves amino acids 121-249. These amino acids are arranged as β -sheets, which are connected to each other by β -turns. The β -turns contains hydrophilic amino acids and are exposed to the surface of the trimers. Based on the available crystallographic data, the alanine at position 167 and phenylalanine at 209 are two strong candidates for mutations to increasing the solubility of AHSV VP7(T13), and therefore could demolish the crystal formation of AHSV VP7(T13). The C-terminal helix 9 might also have an impact on the solubility of VP7(T13), as the C-terminus of AHSV VP7(T13) is more hydrophobic than BTV VP7(T13) (Monastyrskaya *et al.*, 1997). The presence of a large hydrophilic area composed of strand β C and η 1 (aa 168-178, including the RGD motif) of the top domain of one monomer, and the C-terminal helix 9 of the adjoining monomer on the surface of VP7(T13) trimers (BTV and AHSV), have been indicated (Basak *et al.*, 1996).

1.6.4. Incorporation of the three minor proteins within CLPs

Co-expression of VP1(Pol) and/or VP4(Cap) and/or VP6(Hel), using baculovirus vectors, together with VP3(T2) and VP7(T13), demonstrated that each of the minor proteins could be encapsidated within the derived BTV CLPs. When VP7(T13) trimers were removed from CLPs containing VP1(Pol), the derived subcores consisted only of VP3(T2) and VP1(Pol), demonstrating that VP1(Pol) interacts with VP3(T2) (Loudon & Roy, 1991). Similar results were obtained with VP4(Cap) and VP6(Hel) and for combinations of all three minor proteins (Le Blois *et al.*, 1991). However, unlike VP1(Pol) or VP4(Cap), VP6(Hel) was only poorly incorporated into the CLPs. Since VP6(Hel) is a highly basic protein and readily associates with ssRNA or dsRNA, it is possible that VP6(Hel) chaperones the incorporation of RNA into particles and is only poorly incorporated in the absence of RNA (Roy *et al.*, 1990; Hayama and Li, 1994).

It seems likely that the proper organisation of the RNA for the efficient transcription requires that it be laid down within a pre-existing protein shell. The limited size of the pores in the assembled

subcore would, however, prevent entry of the enzymes. It therefore seems probable that the enzymatic components attach to VP3(T2), so that upon formation of the complete subcore there is a transcription complex at each of the 12 fivefold axes (Grimes *et al.*, 1998; Gouet *et al.*, 1999). Electron density maps at lower resolution of BTV cores indicated that the minor structural proteins associated with transcription of the viral genome (transcriptase complex or TC), is positioned directly under the icosahedral 5-fold apices (Grimes *et al.*, 1998; Stuart *et al.*, 1998; Gouet *et al.*, 1999). It is therefore considered possible that there could be 10 or 12 copies of VP1(Pol). VP4(Cap) forms dimers (Devi *et al.*, 1998; Ramadevi *et al.*, 1998) and may therefore be present as either 20 to 24 copies per particle. VP6(Hel) may form hexamers (Stauber *et al.*, 1997) and may be present as 60 to 72 copies per particle. In the orbivirus core, derived from the intact virus, ten of these complexes will be intimately associated with one of the ten segments of the genomic dsRNA. This is in agreement with the data for BTV where four layers of electron density line the inside of the VP3(T2) shell and are closely associated with the TC. This demonstrates that genomic RNA is packaged in a rather structured way, which follows, to a considerable extent, the icosahedral symmetry of the core. This order is imposed by the VP3(T2) layer which possesses, on its inner surface, shallow grooves that seem to form tracks along which the dsRNA lie (Grimes *et al.*, 1998; Stuart *et al.*, 1998). Specific RNA/protein interactions are evident at only two points in the icosahedral VP3(T2) unit, which may facilitate the movement of RNA within the core, for example during transcription (Grimes *et al.*, 1998; Gouet *et al.*, 1999).

Stuart *et al.* (1998) suggested a model for the packaging of the ten segments dsRNA genome within the core so that it remains sufficiently fluid for efficient, repeated and independent transcription. They suggested that each dsRNA segment is associated with a TC complex at the 5-fold apices and is wound around this TC, until it hits a neighbouring RNA segment. At this point due to steric hindrance it may flip down and inward to form the next layer, spiralling back towards the TC. Further switching would lay down the third and fourth layers. This model is also in agreement with some recent observations of other members of the family *Reoviridae* made by cryo electron microscopy (Moss & Nuttal, 1994; Prasad *et al.*, 1996).

1.6.5. Three-dimensional structure of virions and virus-like particles

Image analysis of cryo-electron micrographs revealed well-ordered morphology of the complete virion (Hewat *et al.*, 1992) also displaying icosahedral configuration (86 nm in diameter). The reconstruction revealed a well ordered morphology that contrasts sharply with that deduced by conventional negative-staining methods. The two proteins of the outer capsid have distinctive shapes, one is globular and almost spherical, the other is sail-shaped. The globular VP5 proteins, 120 in number (although biochemical data suggest a total of 360 copies of VP5, indicating that they

may be arranged as trimers (Stuart *et al.*, 1998b), sit neatly in the channels formed by each of the six-membered rings of the VP7(T13) trimers of the core. The sail-shaped spikes, which project 4 nm beyond the globular proteins, are located above 180 of the 260 VP7(T13) trimers and form 60 triskelion-type motifs which cover all but 20 of the VP7(T13) trimers. It is likely that these spikes are the VP2 haemagglutinating and neutralisation antigens, and that the globular proteins are VP5. The two proteins appear to form a continuous layer around the core, except for holes on the fivefold axis. This differs markedly from the structure of the rhesus rotavirus and mammalian reoviruses, which have a more porous structure.

For the synthesis of VLPs, a quadruple gene expression vector was used to synthesise the BTV VP2, VP3(T2), VP5 and VP7(T13) proteins. The expressed proteins assembled into virtually homogenous double capsid particles. Three-dimensional reconstruction of VLPs at 55 Å resolution revealed that the structure of the VLP (86 nm in diameter) is comparable to that of authentic virions. The VLPs exhibit essentially the same basic features and full complement of the four proteins (French *et al.*, 1990; Hewat *et al.*, 1994; Roy, 1992; Roy, 1995).

1.7. DISEASE PREVENTION AND CONTROL: VACCINATION FOR PROTECTION AGAINST VIRAL INFECTION

The main objective of orbivirus vaccination is not to prevent virus infection of the individual animals but to prevent the transmission of the pathogen. In order to achieve this, a vaccine needs to display certain prerequisites. An effective vaccine is one that provokes levels of immunity at the appropriate site, of adequate duration, and of relevant nature, i.e. humoral, cytotoxic T cell or T-helper cell responses (Roitt, 1994).

Protection against viral disease through vaccination can be accomplished by using a live attenuated virus vaccine, an inactivated virus or virus subunits. A subunit vaccine can either be derived from infectious material or produced by genetic engineering involving specific gene expression, or synthetic peptides representing neutralisation epitopes (Roy *et al.*, 1990). Vaccination has played an important role in the diminishing occurrence of AHS in South Africa by reducing the number of susceptible horses. The control of AHSV has thus far been effected by a variety of vaccines (Coetzer & Erasmus, 1994). Annual vaccination of horses is the most practical prophylaxis, with horses that have received three or more courses of immunisation being well protected against the disease (Coetzer & Erasmus, 1994). These vaccines have certain disadvantages, which include a danger of causing the disease it is supposed to protect against.

1.7.1. Conventional live attenuated and inactivated virus vaccines

At present, both BT and AHS are controlled predominantly by annual vaccinations with polyvalent live attenuated vaccines. All AHSV serotypes are distributed throughout South Africa, therefore the use of a polyvalent vaccine is necessary to protect horses in most parts of southern Africa. The current vaccine of choice is one in which live, polyvalent, low virulence, large plaque variants of AHSV are passaged and attenuated (Coetzer & Erasmus, 1994). The AHSV vaccine strains are attenuated by serial passage in Vero (African green monkey kidney) cell cultures (House *et al.*, 1992; House, 1998). These vaccines were based on the finding that those viruses producing large plaques were avirulent (Erasmus, 1972). The vaccine currently in use in South Africa contains 8 distinct serotypes, administered as two quadrivalent doses (one containing serotypes 1,3,4 and 5 and the second serotypes 2,6,7 and 8). The doses are administered 28 days apart and precautions are advised to minimise exposure and stress (House *et al.*, 1992; Onderstepoort Veterinary Institute). Serotype 9 is not included in the vaccines due to the fact that it is afforded some cross-protection resulting from the inclusion of serotype 6 in one of the vaccines. According to Coetzer and Erasmus (1994), cross-neutralisation exists between serotypes 1 and 2, 3 and 7, 5 and 8 and 6 and 9. Annual re-vaccination is recommended because of the inability of the polyvalent mixtures to always generate protection against all serotypes. Full protection against all serotypes is usually expected after 3 to 4 years.

Although these vaccines have been widely and effectively applied, the use of live attenuated polyvalent vaccines does involve risks and inherent deficiencies. Besides the need to identify naturally attenuated strains, or to artificially create attenuation (resulting in high production costs), and the inconvenience of repetitive inoculations, immunological interference between component serotypes in polyvalent vaccines may result in the development of incomplete immunity. In addition, there is the risk of viral reassortment and recombination between attenuated and virulent strains giving rise to new strains, and the potential problem of reversion to virulence (Oberst *et al.*, 1987; Samal *et al.*, 1987; Stott *et al.*, 1987; Katz *et al.*, 1990; Stone-Marschat *et al.*, 1996). The development of viremia has also been reported, with the danger of infection of vectors. Furthermore, modified live vaccines may cause immune suppression and commonly induce latent infections. They have been associated with abortions in late gestation animals, ovarian lesions and infertility (van Drunen Littel-van den Hurk *et al.*, 1993; Yancey, 1993) or foetuses with teratogenic defects (Van Dijk, 1993). Such vaccines have also been implicated in vaccine-induced epizootics (Yancey, 1993; Brown, 1992; Coetzer & Erasmus, 1994). The immune response generated after vaccination with modified live vaccines cannot be distinguished from those elicited by natural infection (House, 1998). Further side-effects may include fatal encephalitis, which is characterised by blindness and neurological disorders (Coetzer & Erasmus, 1994).

Non-replicating vaccines have theoretical advantages over live attenuated virus vaccines due to the absence of active replicating virus, eliminating many of the risks and deficiencies associated therewith. Efforts have thus been made to develop effective inactivated vaccines. Experimental inactivated viral vaccines (IVV) were reported as early as 1966 but the process was never commercialised, although a IVV for AHSV-4 was developed and used experimentally during the 1987-1990 epizootic in Spain. The efficacy of the AHSV-4 IVV as a single dose vaccine was evaluated and indicated that a single dose of IVV would protect horses from severe clinical signs and death but not necessarily from the development of a viraemia. However, vaccination with two doses of IVV not only give full protection but also prevent the infection of insect vectors, which is a critical consideration for eradicating AHSV from non-enzootic countries (Hassanian et al., 1992; House *et al.*, 1994; House, 1998). The production of inactivated vaccines is complex and expensive. These vaccines do not confer long lasting immunity thus require multiple doses. In addition the afforded protection is not always complete and cross-protection of immunised animals is restricted to viruses of the same serotype. In some cases outbreaks have reportedly been caused by escape of virus from the use of improperly inactivated vaccines. However, many procedures for the effective and reliable inactivation of viruses, both chemical and genetic, have been developed, which should eliminate this problem (House & House, 1989; House et al., 1992b; Burrage & Laegreid, 1994). Another major drawback of inactivated vaccines is the hypersensitisation that has been reported when sheep previously vaccinated with inactivated BTV are challenged with virulent virus (Stott et al., 1985).

1.7.2. Recombinant subunit and peptide vaccines

Recent advances in recombinant DNA technology have prompted a new era in vaccine development, offering new possibilities of preparing genetically engineered vaccines without the need to grow the pathogenic organism. This new direction in vaccine development avoids the difficulties and the risks associated with live virus, or preparation of killed virus vaccines. Proposed vaccines based on this new technology include cloning of portions of the viral genome into a vector that can be administered or used to express viral proteins, the formation of empty capsids lacking the ability to replicate, the production of synthetic peptide vaccines and genetic immunisation. Attention has been focussed on identifying the relevant proteins and sequences involved in protective immune responses to viral infection and on systems to produce and present these proteins, or protein sequences, to elicit the required protection.

1.7.2.1. AHSV antigenic outer capsid proteins and neutralisation domains

In the case of BTV, vaccination with baculovirus-derived BTV VP2 was found to fully protect sheep against virulent homologous BTV, with complete absence of clinical signs and post-challenge viraemia (Inumaru & Roy, 1987; Roy *et al.*, 1990). The outer capsid protein, VP2, of BTV and AHSV induces serotype-specific neutralising antibodies that protect animals fully against virulent virus challenge mediated by a humoral immune response (Huismans *et al.*, 1987; Burrage *et al.*, 1993; Martinez-Torrecedrada & Casal, 1995). Subunit vaccines for AHSV and BTV are being developed as an alternative to the existing live attenuated polyvalent vaccines including VP2, mixtures of VP2 and VP5 and VLPs (Roy *et al.*, 1990; Pearson & Roy, 1993; Van Dijk, 1993; Martinez-Torrecedrada *et al.*, 1996). Animals vaccinated with these subunit preparations can also be distinguished from naturally infected animals, which would simplify international movement of horses. Since it was demonstrated that VP2 alone is sufficient to protect horses against AHSV, studies aimed at developing a subunit vaccine against AHSV have been focused mainly on VP2 (Roy *et al.*, 1996; Stone-Marchat *et al.*, 1996). High levels of expression could be achieved when recombinant VP2 was expressed in a baculovirus system, and VP2 of AHSV-4 was reported to be able to protect horses when expressed in this manner (Roy *et al.*, 1996). Although, this suggests that a recombinant baculovirus, expressing VP2 of AHSV (like BTV), may be used to produce antigenic protein for a suitable subunit vaccine, vaccination studies using baculovirus expressed VP2 proteins of other serotypes, experienced complications due to protein aggregation (Du Plessis *et al.*, 1998). These include limited protection caused by insufficient immunogenicity of the recombinant proteins despite the high level of expression. It was thought that the insufficient immunogenicity of the proteins could possibly be caused by the insoluble nature of the baculovirus expressed VP2 (Du Plessis *et al.*, 1998; Venter *et al.*, 1999). Vreede & Huismans (1994) and Martinez-Torrecedrada *et al.* (1994), also proposed that the soluble form of VP2 has a toxic effect on insect cells, which resulted in very low levels of soluble VP2. Since solubility is important for a protein to act as an effective immunogen, the approach of using VP2 as a subunit vaccine is currently prevented by the lack of producing high yields of soluble VP2. Previous studies with BTV indicated that the presence of VP5 together with VP2 enhances the neutralisation antibody and protective responses against virulent BTV challenge. It has been postulated that VP5 improves the conformation of VP2 when administered together (Roy *et al.*, 1990).

An alternative approach is to identify important epitopic regions on VP2 of AHSV by mapping the exact position of the epitopes using overlapping peptides or phage display libraries. A linear neutralising epitopic domain had been identified on VP2 of AHSV-4 by screening overlapping truncated protein fragments of VP2 with neutralising monoclonal antibodies as well as polyclonal neutralising sera, and by using these fragments to elicit neutralising antibodies in mice (Martinez-

TorreCuadrada *et al.*, 1994; Martinez-TorreCuadrada & Casal, 1995). The study revealed that the major antigenic domain of AHSV-4 VP2 is located in the central region of the protein and that several neutralisation sites are located within this domain. A region between amino acids 253 and 413 was able to elicit consistently high titres of neutralising antibodies. However, this recombinant polypeptide was not able to compete with virus particles for binding of neutralising antibodies in serum from infected or vaccinated horses, which indicates that this region is not immunodominant in infected horses (Martinez-TorreCuadrada & Casal, 1995). A similar approach to epitope mapping was applied to VP2 of serotype 9 using baculovirus expression. A region from residues 252 to 486 was recognised to contain a linear epitope of approximately 33 amino acids. This region that stretches from amino acids 369 to 403 is located in the same region as a neutralising linear epitope identified on AHSV-4 VP2 and is implicated in virus neutralisation (Venter *et al.*, 1999; TorreCuadrada & Casal, 1995). The polypeptides identified in each of these studies were insoluble and thus not ideal immunogens.

1.7.2.2. Protection afforded by biosynthetic particulate structures

VLPs synthesised by recombinant baculoviruses mimic the native virion in size and appearance. The VLPs are not only biologically, but also immunologically similar to the virion. VLPs exhibit high levels of haemagglutination activity, similar to those of authentic BTV and are capable of eliciting protective immune response, but are not infectious since the particles lack genetic material. BTV VLPs were investigated for their vaccine potential in sheep and proved to be highly immunogenic. Antibodies raised to the expressed particles contain high titres of neutralising activity against the homologous BTV serotype (French *et al.*, 1990; Roy *et al.*, 1992). Long lasting protection against homologous BTV challenge has been provided by vaccination with VLPs, as well as some preliminary evidence for cross protection, depending on the amount of antigen (Roy *et al.*, 1994). It has been illustrated that BT VLPs are highly immunogenic, even at low doses compared to VP2 alone or mixtures of VP2 and VP5. Assuming that VP2 plays the primary role in humoral protection, the results implied that 25-50 fold less VP2 was sufficient for protection when presented on VLPs compared to its individual effect or in conjunction with VP5 (Roy *et al.*, 1990 & 1992). It has been suggested that the VP3(T2) and VP7(T13) provides the necessary scaffold for the correct conformational presentation of the relevant epitopes on VP2, which elicits humoral immune response and that any of the 4 BTV capsid proteins might have a direct role in eliciting cell-mediated immunity. Furthermore, both VP2 and VP5 are present (Pearson & Roy, 1993; Roy & Sutton, 1998). It is therefore of critical importance to produce AHS VLPs, which may prove to be an effective and safe recombinant subunit vaccine and will also allow differentiation between vaccinated and naturally infected horses.

BTV CLPs have been demonstrated to be formed by the co-expression of VP3(T2) and VP7(T13) in recombinant baculovirus infected insect cells (French & Roy, 1990). Sheep vaccinated with BTV CLPs and challenged with virulent virus developed only limited clinical signs of disease and all recovered fully. Preliminary serological investigations indicated that immunisation with CLPs did not induce neutralising antibodies, nor did it affect the neutralising antibody response to the challenge virus (French *et al.*, 1990). The finding that CLPs can induce protection in the absence of neutralising antibodies suggested the involvement of a cell-mediated response, but this remains to be investigated (Van Dijk, 1993). Vaccination trials with BT CLPs resulted in partial protection in the absence of neutralising antibodies, suggesting the involvement of cell-mediated response (Pearson & Roy, 1993; Roy & Sutton, 1998). Although immunisation with CLPs has not yet been reported in AHSV, it was recently demonstrated that mixtures of VP2, VP5 and VP7(T13) of AHSV conferred protection in horses against virulent AHSV challenge, although only low levels of neutralising antibodies were induced (Martinez-Torrecuadrada *et al.*, 1996). It has been suggested that VP5 and VP7(T13) could possibly contain T-cell epitopes, which could enhance the immune response.

Wade-Evans *et al.* (1997) demonstrated that AHSV VP7(T13) crystals protect mice against lethal, heterologous serotype challenge, not involving antibody-mediated immune response. This correlates with previous suggestions for BTV that cell-mediated immunity may play an important role in the development of a protective immune response. Since VP7(T13) is the major serogroup specific antigen of AHSV, and thus also the serotype cross reactive antigen, these results may indicate that VP7(T13) crystals may be effective as a prophylactic subunit vaccine against all serotypes of AHSV (Wade-Evans *et al.*, 1997).

However, the production of AHSV subunit vaccines may become a reality in the not-so-distant future, which would greatly alleviate these problems. An advantage of subunit vaccines will be that they make it possible to distinguish between horses that have been vaccinated and those that have been infected with the virus itself. Increasing use of molecular biological analysis methods and recombinant DNA technology in recent years has accelerated our knowledge of the functions and structures of the orbivirus proteins, including those of AHSV and may thus find direct application in the production of a suitable subunit vaccine and also in the production of virulence or attenuated markers.

1.8. EXPRESSION SYSTEMS FOR RECOMBINANT PROTEINS

Our ability to achieve these objectives depends on high level expression of the relevant heterologous AHSV genes. There are five major expression systems that are commonly available, namely *Escherichia coli*, *Bacillus subtilis*, yeast, baculovirus and mammalian expression systems (Goeddel, 1991). Proteolysis represents one of the most significant barriers to heterologous gene expression in any organism. Another problem results from the expression of certain recombinant proteins that turn out to be toxic to the cell. The only possible solutions are to switch to a different expression system, to target the toxic proteins into an organelle where its toxicity is no longer manifested.

All expression systems have advantages and disadvantages that should be considered when choosing a suitable expression system. Expression in *E. coli* is neither time-consuming, nor expensive and some strains can produce 30% of their total protein as the expressed gene product. However, eukaryotic proteins expressed in *E. coli* are not properly modified. Proteins expressed in large amounts in *E. coli* often precipitate into soluble aggregates or inclusion bodies, from which they can only be recovered in the active form by solubilisation in denaturing agents followed by careful renaturation. The reducing environment present in *E. coli* does not permit cysteine-rich proteins to form the disulphide bonds required for proper conformation. However, intracellular production of heterologous proteins in *E. coli* is possible even for those proteins that must be oxidised for activity (Gold, 1991).

Yeast are unicellular micro-organisms and many of the manipulations commonly used in bacteria can also be readily applied to yeast. Yeast offers many advantages of both prokaryotic and eukaryotic systems. Yeast grow rapidly, achieve high cell densities and are able to perform post-translational modifications. They can be propagated on simple defined media and transformed with a variety of either self-replicating or integrating plasmid vectors. Yeast being eukaryotic, possess much of the complex cell biology typical of multicellular organisms, including high compartmentalised intracellular organisation and an elaborate secretory pathway which mediates the secretion and modification of many host proteins. However, some proteins are rapidly degraded either during or shortly after synthesis. Others are lost during cell breakage and subsequent purification (Emr, 1991).

Progress in mammalian cell expression systems has been astonishing in the last few years. Mammalian expression systems have several advantages for the expression of higher eukaryotic proteins. Expressed proteins are properly modified and they almost always accumulate in the correct cellular compartment. However, mammalian expression systems are expensive, complicated and more time-consuming than any of the other systems described here (Levinson, 1991).

1.8.1. Baculovirus expression vector system (BEVS)

Since its introduction in 1983, the BEVS technology has become one of the most versatile and powerful eukaryotic vector systems for recombinant protein expression. A wide variety of genes from viruses, fungi, plants and animals have been expressed in insect cells infected with recombinant baculoviruses (Luckow & Summers, 1988; Maeda, 1989; Luckow, 1991; King & Posse, 1992). *Autographica californica* nuclear polyhedrosis virus (AcNPV) is a proven vector for high level expression of heterologous genes in insect cells (O'Reilly *et al.*, 1992).

Owing to the large size of the baculovirus genome (about 130 kb), the BEVS relies on *in vivo* recombination to replace a viral allele, usually the dispensable polyhedrin gene of AcNPV, with the gene of interest cloned into a suitable transfer vector. Transfer vectors contain a site for foreign gene insertion, downstream of a proficient promoter of a wild type viral protein expendable for virus replication, and flanked by viral sequences homologous to the part of the baculovirus genome where the gene is to be inserted. Subsequent co-transfection of insect cells with infectious baculoviral DNA and recombinant transfer plasmid DNA allows cell-mediated allelic replacement of the target viral gene with the plasmid-borne foreign gene through homologous recombination. The percentage of recombinant viruses obtained by this recombination event has been reported to constitute 0.1-1% of the progeny viruses (Kitts *et al.*, 1990; Kitts & Possee, 1993; Zuidema *et al.*, 1990; Anderson *et al.*, 1995). Numerous modifications of this system have been made to increase the frequency of recombination and improve the ease with which recombinants can be selected (Davies, 1995; Miller, 1989 & 1993). However, all methods involve repeated rounds of plaque purification to ensure that recombinant virus is not contaminated with wild type baculovirus. This is time consuming and costly.

BEVS has a number of advantages, eg. extremely high rate and duration of the expression of recombinant proteins, the antigenic, immunogenic and functional similarity of the expressed proteins to their authentic counterparts, the applicability of the system to monolayer or suspension culture and the post-translation modification of proteins in a manner similar to those of mammalian cells. Baculoviruses are essentially non-pathogenic to mammals and plants. This system has proven to be successful in expressing genes as non-fusion proteins, therefore, genes using their natural, translation initiation codon and N-terminal sequences. Although N-terminal fusions may increase the stability of some proteins, the biological activity of many proteins is adversely affected by additional sequences at the N-terminal (Miller, 1988; Yong Kang, 1988; O'Reilly *et al.*, 1992).

Glycosylation of proteins in the baculovirus system has been regarded as relatively successful. This is at first sight surprising, as notable differences exist between the glycosylation pathways of insects

and higher eukaryotes (Davies, 1995). Post-translational modifications in BEVS such as phosphorylation and glycosylation mimic those modifications by mammalian cells.

Synthesis of proteins involved in the formation of complex structures, in eucaryotic cells by an expression vector provides an opportunity to investigate macromolecular interactions under more natural, intracellular conditions. The baculovirus expression system has been exploited extensively over the past few years to investigate the assembly of the major BTV capsid proteins into core-like particles and virus-like particles via co-expression of the relevant genes in insect cells (French *et al.*, 1990; French & Roy, 1990). The productivity and flexibility of the insect baculovirus expression vector system and the ability of the baculovirus genome to incorporate and express large amounts of foreign DNA have permitted this system to be used for the simultaneous expression of multiple foreign genes in a single insect cell (Bishop, 1992). The foreign genes are placed under control of the polyhedron or p10 promoters, which are both non-essential for viral morphogenesis and strong and active in the very late phase of infection. The p10 promoter is responsible for the high level synthesis of a non-structural protein in wild type baculoviral infected cells (Balyaev & Roy, 1993). It is activated in the very late occlusion phase of virus replication, and appears to have a similar relative strength to the polyhedrin promoter (Weyer *et al.*, 1990). The function of the p10 protein is unknown, but it may be involved in host cell lysis (Weyer *et al.*, 1989). The promoters are arranged in opposite orientations to minimise the possibility of homologous sequence recombination and excision of the foreign genes.

BAC-TO-BACTM baculovirus expression:

Recently, Luckow and co-workers (1993) introduced a novel baculovirus expression system (BAC-TO-BACTM) which permits rapid and efficient generation of recombinant viruses by site-specific transposition of a DNA cassette into a baculovirus shuttle vector (bacmid), propagated in *E. coli*, rather than homologous recombination in insect cells.

The bacmid system utilises transposon 7-mediated transposition in *E. coli* to produce recombinant AcNPV (Davies, 1994; Leusch *et al.*, 1995). The bacmid is a recombinant baculovirus genome (AcNPV) containing a mini-F replicon inserted into the polyhedrin locus, a kanamycin resistant marker, and a mini *attTn7* (target site for bacterial transposon TN7) inserted within a *lacZ α* complementation region. Therefore the bacmid can replicate in *E. coli* as a plasmid and can also infect susceptible insect cells. The donor plasmid contains the polyhedrin promoter to drive expression of the foreign gene, flanked by left and right ends of Tn7, a SV40 poly(A)-sequence and a multiple cloning site downstream of the original ATG of the polyhedrin gene, which has been mutated to ATT. Therefore, to successfully express a protein, the foreign DNA fragment must contain its own ATG followed by an ORF. To generate a recombinant baculovirus, the gene of interest is cloned into the donor vector downstream of the polyhedrin promoter. A dual expression

transfer vector, pFastbac-Dual, which facilitates the introduction of two heterologous foreign genes inserted at unique multiple cloning sites into a single recombinant virus, is also available. Both foreign proteins are then expressed, under control of two strong very late promoters respectively, the polyhedrin and p10 promoters.

The recombinant donor plasmid is then used to transform *E. coli* DH10Bac cells, containing the bacmid and a helper plasmid, which provides transposition functions in *trans*. The transposition event inserts the gene of interest and polyhedrin promoter into the bacmid. Composite bacmid DNA is transfected into insect cells giving rise to recombinant baculoviruses expressing the gene of interest. The characteristics of the BAC-TO-BAC™ system reduce the time to identify and purify a recombinant virus from 4-6 weeks to 7-10 days. This method permits rapid and simultaneous isolation of multiple recombinant viruses and is particularly suitable for expression of protein variants for structure/functional studies.

1.9. SUMMARY AND AIMS

As discussed, the current AHS vaccine, although effective, does involve certain risks and has some inherent deficiencies. At least some of these concerns, including the induction of latent infections and the potentially serious problems of reversion to virulence and vaccine induced epizootics, may be overcome through the development of recombinant vaccines. In the development of any recombinant vaccine, it is mandatory to identify and characterise all the antigens required to stimulate a protective immune response. A disadvantage of a recombinant peptide vaccine is the difficulty in presenting the protective protein, protein fragments or small peptides in their correct three-dimensional structure for an effective immune response. Therefore, it may be found essential to use a particulate delivery system for the display of immunodominant epitopes to evoke an immune response. This focuses the attention on the possible use of AHSV particulate structures for the presentation of antigens or neutralising epitopes to the immune system of the recipient animal.

From the literature review, it is clear that AHSV is associated with three characteristic structures, which are highly immunogenic and could be used as particulate delivery systems for immunologically important determinants. The unique virus-specified tubular structures, composed of NS1, observed in the cytoplasm of orbivirus-infected cells, were proven to be an effective carrier for foreign epitopes, in the case of BTV (Mikhailov *et al.*, 1996). Secondly, AHSV VP7(T13) is highly hydrophobic and aggregate into distinctive hexagonal crystals when expressed in the absence of the other AHSV proteins. These crystals elicit a strong humoral immune response in an animal model (Wade-Evans *et al.*, 1997 & 1998). Thirdly, empty virions or particles that simulate the virion surface should provide a safe way of stimulating protective immunological memory.

The long-term aim of this investigation is to obtain more information on the morphology, biochemical and biophysical properties and the factors involved in the polymerisation of these three AHSV-associated structures and their possible use as peptide delivery systems. The foundation of such an investigation is based on the availability of the genes, knowledge about the gene sequences and a system for producing the required amounts of the proteins. The gene segments encoding the major structural proteins were cloned and characterised previously, but no functional DNA copy of the NS1 encoding segment of AHSV was available.

For these reasons this project was focussed on the following short-term objectives:

Primary objectives:

1. Cloning and characterisation of the AHSV-6 NS1 gene, analysis of the morphological and biophysical properties of the deduced protein and identification of epitope insertion sites.
2. Verification of the interaction of the four major structural proteins to form core-like or virus-like particles and analysis of the morphology of the assembled particles.
3. Modification of the hydrophilic regions of the top domain of AHSV-9 VP7(T13) by means of site-directed insertion mutagenesis and investigation of the effect of additional sequences on the trimerisation, solubility, crystal formation and CLP formation of VP7(T13) as well as the length of inserts tolerated by VP7(T13).

The research strategies for obtaining the primary objectives involved the following:

1. Cloning and sequencing of a full-length cDNA copy of the NS1 gene. Identification of hydrophilic regions which may potentially be used for the insertion of foreign epitopes. Expression of the AHSV-6 NS1 gene with a view to obtain large quantities of the NS1 protein, in the absence of the other viral components, for studies regarding NS1 structure and biophysical properties (see chapter 2).
2. The simultaneous expression of the major proteins of the core particle (VP3(T2) and VP7(T13)) or virus particles (VP2, VP3, VP5 and VP7) in insect cells in order to synthesise core-like (CLPs) or virus-like particles (VLPs). Structural analysis, carried out using electron microscopy (see chapter 3).
3. Insertion mutants of AHSV VP7(T13) will be prepared by means of PCR based site-directed insertion mutagenesis. Previously identified neutralising epitopes of AHSV-9 VP2 will be cloned into the modified regions of VP7(T13). The effect these modifications have on trimer, crystal and CLP formation of VP7(T13) was investigated (see chapter 4).

CHAPTER 2

CHARACTERIZATION OF TUBULAR STRUCTURES COMPOSED OF NONSTRUCTURAL PROTEIN NS1 OF AFRICAN HORSESICKNESS VIRUS EXPRESSED IN INSECT CELLS

2.1. INTRODUCTION

Little is known about the exact roles of the three non-structural proteins in the replication cycle of orbiviruses. Data suggest that these proteins are involved in the process of viral morphogenesis, leading to viral assembly and release. There is as yet no evidence for a specific function of NS1 tubules in virus replication, although it has been proposed that NS1 may play a role in the transport of mature virus particles from the virus inclusion bodies to the cell membrane where NS3 is involved in virus release. Studies have shown that the structure, size and biophysical character of the NS1 tubules differ significantly among AHSV, BTV and EHDV (Huisman & Els, 1979). However, the function of the tubules may be conserved because their synthesis is a characteristic feature of the replication cycle of all orbiviruses investigated thus far. NS1 is potentially useful as a diagnostic antigen since the presence of specific antibodies is an indication that the virus has replicated in the host. It is, however, not antigenically unique and cross-reacts serologically with the analogous protein of the related epizootic haemorrhagic disease virus (EHDV) (Richards *et al.*, 1988).

The baculovirus expression system enabled significant biochemical and structural analysis of BTV and EHDV NS1 tubules in the absence of other viral components (Urakawa & Roy, 1988; Nel & Huisman, 1991). BTV NS1 is a highly hydrophobic protein and particularly rich in cysteine residues (Lee & Roy, 1987; Nel *et al.*, 1990). BTV and EHDV NS1 proteins are highly ordered structures, most likely due to the large amount of conserved cysteine residues and the major hydrophilic and hydrophobic regions scattered throughout the protein (Fukusho *et al.*, 1989; Nel *et al.*, 1990). Monastyrskaya *et al.* (1994) have revealed several conserved elements in the protein which are necessary for tubule formation, with cysteine residue at positions 337 and 240 and the conserved carboxyl- and amino-terminals shown to be of particular importance. The amino acid sequence of NS1 and the structure and biophysical character of the tubules differ significantly among AHSV, BTV, EHDV and BRDV (broadhaven virus).

Three dimensional studies of the BTV NS1 tubules indicated that each tubule has a diameter of 52.3 nm and is composed of a coiled ribbon of NS1 dimers with 22 dimers per helix turn (Hewat *et al.*, 1992). Very little is known about the structure of the AHSV tubules except that they appear to be morphologically different from EHDV and BTV tubules (Huisman & Els, 1979). Although the NS1 gene of AHSV-4 and 9 has been sequenced there are no reports on the structure and biochemical/biophysical properties of AHSV tubules. From the literature review it is clear that many of the details of the process of orbivirus replication, morphogenesis and assembly still need to be elucidated. The non-structural proteins are thought to play important roles in these processes. The role of NS1 and of the tubules in these processes is still obscure.

Recently, Mikhailov *et al.* (1996) have shown that NS1 of BTV can be utilised as an immunogen delivery system, as they had succeeded in inserting sequences ranging from 44 to 116 amino acids in length at the C-terminus of BTV-10 NS1. All the chimeric constructs still possessed the ability to form tubular structures while carrying the foreign antigenic sequences when expressed as recombinant baculoviruses. Interestingly, when insect cells were co-infected with three recombinant baculoviruses expressing the NS1 chimeric proteins with different epitopes, they simultaneously assembled into the same tubule. This has important implications for using NS1 tubules for the structured delivery of multiple epitopes. Two unique antigenic regions of BTV NS1 were identified, using a phage display library, one of which could be effectively mimicked by a 28 residue synthetic peptide (Du Plessis *et al.*, 1995). This peptide did not cross-react with an antiserum directed against NS1 of EHDV and was identified to be located at position 474 to 502.

In order to obtain more information on the characteristics of AHSV NS1. This chapter describes the cloning and characterisation of the NS1-encoding gene of AHSV serotype 6, as well as a comparison with the cognate gene and derived amino acid sequence of AHSV-4 and -9, BTV and EHDV. The sequence data will be used to identify regions that could be utilised for the insertion of foreign epitopes. The NS1 protein of AHSV-6 was also expressed by means of a baculovirus recombinant producing AHSV-specific tubules for elucidation of the structure, biochemical character and stability of these tubules.

2.2. MATERIALS AND METHODS

2.2.1. Materials

The pBS cloning and expression vector and pUC18 were obtained from Stratagene and Boehringer Mannheim, respectively. G-tailed *Pst*I cut pBR322 was purchased from GIBCO BRL. The BAC-TO-BAC™ baculovirus expression system was obtained from Life Technologies, Inc. Restriction endonucleases, calf intestinal alkaline phosphatase (CIP), RNase A, T4 DNA ligase, Klenow polymerase, dNTPs, DNA molecular weight marker II (MWII) and T7 RNA polymerase were purchased from Boehringer Mannheim. *Hae*III-digested ϕ X 174 DNA, T3 RNA polymerase and Taq polymerase were obtained from Promega. The GeneClean™ DNA purification kit was purchased from Bio 101 Incorporated. United States Biochemical Corporation (USB) supplied the sequenase™ Version 2.0 sequencing kit. The following radioisotopes were supplied by Amersham: [α -³⁵S]dATP (1 mCi/ml; > 6000 Ci/mmol), [α -³²P]dCTP (1 mCi/ml; > 400 Ci/mmol), [γ -³⁵S]methionine (15 mCi/ml; 1000 Ci/mmol). Amersham also supplied Hybond™C, N and N⁺ nitrocellulose membranes. Cronex MRF 31 X-ray film was supplied by Protea Medicals. A nick translation kit was supplied by Promega. Rainbow™ protein molecular weight marker (MW 14 300 - 200 000 Da), human placental RNase inhibitor (HPRI) and nuclease treated message dependent rabbit reticulocyte lysate was also purchased from Amersham. The Lipofectin™ and Cellfectin™ reagents were supplied by GIBCO BRL. Onderstepoort Veterinary Research Institute provided Eagle's medium with or without methionine. Grace's insect medium and foetal calf serum were obtained from Highveld Biological. Other chemicals were purchased from Merck, Sigma and Boehringer Mannheim.

2.2.2. Cells and viruses

South African isolates of AHSV serotype 3 and 6 were obtained from the Onderstepoort Veterinary Research Institute (OVI), Onderstepoort, South Africa. AHSV was propagated in monolayers of chicken embryo reticulocyte (CER cells) or baby hamster kidney cells (BHK cells) grown in Eagle's medium supplemented with 5% bovine serum. Wild type and recombinant baculoviruses were propagated and assayed in *Spodoptera frugiperda* (Sf9) cell cultures. The Sf9 cells were supplied by the NERC Institute of Virology and Environmental Microbiology, Oxford, UK. Sf9 cells were grown as monolayers or suspension cultures at 28°C in Grace's medium (Summers & Smith, 1987) supplemented with 10% (v/v) foetal calf serum and antibiotics (penicillin/streptomycin/fungizone).

2.2.3. Partial characterisation of AHSV serotype 9 NS1 gene by *in vitro* expression

A cDNA copy of the segment 5 gene of AHSV-9, encoding the NS1 protein has previously been cloned by Nel and Huismans (unpublished data) by the cloning technique described by Bremer *et al.* (1990). AHSV-9 segment 5 cDNA and PCR gene copies, respectively cloned into pBR322 (pBR-AH9S5cDNA) and the pSPT19 expression vector (T3 orientation; pSPT-AH9NS1) were digested with each of the following restriction enzymes in order to verify the identity of the clones: *Bam*H1, *Hind*III, *Xho*I and *Xba*I. Restriction enzyme digestions were carried out according to the manufacturer's instructions.

Using the original AHSV-9 NS1 cDNA clone (pBR-AH9S5cDNA) and the PCR copy (pSPT-AH9NS1), a NS1 PCR-cDNA chimeric gene was constructed within pSPT19. The restriction enzyme map of the AHSV-9 NS1 gene was used to identify exchange sites close to each terminal, namely *Acc*I (ca. 450 bp from the 5' end) and *Xho*I (ca. 350 bp from the 3' end). To confirm the presence of these sites in both the

cloned cDNA and PCR copies of the gene, each recombinant plasmid was digested with *Accl* and *XhoI* enzymes, individually and simultaneously. DNA fragments of interest were excised from pBR-AH9S5cDNA and pSPT-AH9NS1, respectively containing the cDNA and PCR-tailored copies of the NS1 gene. pSPT-AH9NS1 was simultaneously digested with *Accl* and *XhoI* enzymes to generate a 1.1 kb and 3.9 kb fragments. The ca. 1.1 kb segment 5 cDNA sub-fragment was excised from pBR5cDNA by simultaneous restriction with *Accl* and *XhoI*. The fragments of interest were recovered from a 1% agarose gel and cloned as described in section 2.2.7. The ends were verified by sequencing.

2.2.4. Cloning of AHSV-6 segment 5 cDNA using homopolymeric dC-tailing

The isolation of AHSV serotype 6 viral dsRNA and the synthesis of cDNA were performed by Grant Napier, using the strategy as described by Bremer *et al.* (1990). The double-stranded cDNA fragment of AHSV-6 segment 5, was cloned by a modification (Huismans & Cloete, 1987) of the method described by Cashdollar *et al.* (1982 & 1984). Homopolymeric dC-tails were added to the 3' ends of the cDNA using terminal deoxynucleotidyl transferase (TdT; Gibco BRL) as described by Deng & Wu (1981). The C-tailing reaction (30 μ l) contained 0.1 mM dCTP and 20 μ Ci α^{32} P dCTP (400 Ci/mmol; Amersham) in $1/5$ volume 5x DNA tailing buffer (0.5 M potassium cacodylate pH 7.2, 10 mM CoCl_2 , 1 mM dithiothreitol (DTT)) and 15 U TdT. The reaction was incubated at 37°C for 15 min and the cDNA was purified by the GeneClean™ procedure (section 2.2.8.2). The dC-tailed product was annealed to the dG-tailed *PstI*-cut pBR322 in a DNA thermal cycler. The annealing reaction was carried out in a final volume of 30 μ l containing the purified dC-tailed cDNA incubated with 200-400 ng dG-tailed *PstI*-cut pBR322 (Gibco BRL) in annealing buffer (10 mM Tris-HCL pH 8.0, 150 mM NaCl, 2 mM EDTA) for 5 min at 80°C. The mixture was then subjected for 1 h each at 65°C, 56°C, 42°C and RT to allow annealing. This DNA was then used to transform competent *E. coli* HB101 cells.

2.2.4.1 Preparation of *E. coli* competent cells

The calcium chloride method of preparing competent cells, originally described by Cohen *et al.* (1972), was used, whereby exposure to calcium ions renders cells able to take up DNA, or become competent. The technique is described in Sambrook *et al.* (1989). *E. coli* HE101, JM105 or XL1-Blue cells were routinely used for transformations with pBR322, pUC18 and pBS (Bluescript) plasmid DNA, respectively. An overnight culture of *E. coli* was used to inoculate 100 ml of pre-warmed sterile Luria-Bertani (LB) medium (1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, adjusted to pH 7.4 with NaOH) and the cells were grown at 37°C with continuous shaking to logarithmic (log) phase ($\text{OD}_{550} = 0.5$). Cultured cells (20 ml) were collected by centrifugation at 4 000 rpm for 5 min at 4°C and the pellet was gently resuspended in half the original volume (10 ml) of ice cold freshly prepared 50 mM CaCl_2 and incubated on ice for 30 min. The cells were collected by centrifugation as above and resuspended in $1/20$ of the original volume of CaCl_2 (1 ml). The cells were kept on ice for at least 1 h before transformation.

2.2.4.2 Transformation of competent cells with plasmid DNA

The annealed cDNA/vector mixture was added to 200 μ l of competent *E. coli* HB101 cells and allowed to adsorb for 30 min on ice. The cells were then subjected to a heat-shock of 42°C for 90 s (described by Sambrook *et al.*, 1989), to allow the DNA to enter the cells, and cooled on ice for 2 min. Pre-warmed (37°C) LB-medium (0.8-1.0 ml) was added, and the cells were incubated at 37°C with shaking for 1 h to allow expression of the plasmid-encoded antibiotic resistance. The cells were plated out in aliquots of 100-150 μ l onto 1.2% (w/v) LB-agar plates containing the appropriate antibiotic (12.5 μ g/ml tetracycline hydrochloride (tet) and/or 100 μ g/ml ampicillin (amp)). Plates were incubated overnight at 37°C.

Plates for transformations of recombinant pBR322 plasmids contained 12.5 µg/ml tet. Colonies were replica-plated on amp and tet plates, as ligation of a cDNA insert in the *Pst*I site of pBR322 inactivates the ampicillin resistance gene. Recombinant plasmids were selected on the basis of tetracycline resistance (tet^R) and ampicillin sensitivity (amp^S). Colonies of desired phenotype were picked with sterile toothpicks and grown overnight in 5 ml LB medium with the appropriate antibiotic.

2.2.5. Plasmid DNA extraction and purification

Recombinant plasmid DNA was extracted from liquid cultures of bacterial cells according to the rapid alkaline lysis method (Sambrook *et al.*, 1989) which is a modification of the method originally described by Birnboim & Doly (1979). The following procedure describes mini-prep plasmid purification. A single bacterial colony was inoculated into 3-5 ml LB-medium supplemented with the appropriate antibiotic and incubated overnight at 37°C. Cells in 3 ml of culture were harvested by centrifugation at 12 000 rpm for 2 min in a microcentrifuge. After removing the supernatant the cells were resuspended in 200 µl of ice cold solution 1 (25 mM Tris-HCL pH 8.0, 50 mM glucose and 10 mM EDTA). After 5 min at RT and 2 min on ice, 400 µl of a freshly prepared solution of 0.2 N NaOH, 1% SDS was added, gently mixed and incubated on ice for 5 min, lysing the bacteria and causing denaturation of the proteins as well as the chromosomal and plasmid DNA. To this 300 µl of 3 M sodium acetate (NaAc), or potassium acetate (KAc) pH 4.8 was added and vortexed vigorously, resulting in reannealing of the plasmid DNA and precipitation of the chromosomal DNA and protein. After 10 min on ice, the supernatant was collected by centrifugation at 15 000 rpm for 10 min at 4°C and transferred to a fresh tube for ethanol precipitation of the plasmid DNA. The plasmid DNA was recovered from the supernatants by the addition of two volumes (1.8 ml) 96% ethanol or 0.7 volumes 100% isopropanol (630 µl) and incubated at -20°C for 1 h or at room temperature for 1 h, respectively. The DNA was pelleted by centrifugation and washed once with 500 µl of ice cold 70% ethanol. Pellets were vacuum dried and resuspended in 40 µl 1 x TE buffer (10 mM Tris-HCL, 1 mM EDTA, pH 8.0). The volume of each reagent was adapted to correlate with 200 ml suspension cultures for large scale plasmid extractions. Contaminating low molecular mass RNA was removed by precipitation with half a volume of 7.5 M ammonium acetate and plasmid DNA was finally recovered by ethanol precipitation.

2.2.5.1. Phenol-chloroform purification

Mini-prep samples were deproteinized by phenol-chloroform extraction. TE buffer (1 x) was added to a final volume of 400 µl and mixed with an equal volume of a 25:24:1 solution of phenol:chloroform:iso-amylalcohol followed by centrifugation at 12 000 rpm for 5 min. The upper aqueous phase was removed and extracted twice with a half volume of chloroform. DNA was recovered from the aqueous phase by ethanol precipitation as described earlier, after the addition of 3M Na.acetate pH 7.0 to a final concentration of 0.3 M.

2.2.5.2. RNase-PEG precipitation

Recombinant plasmid DNA obtained from small-scale plasmid extractions were incubated with 1 µl RNase A (10 mg/ml) at 37°C for 20-30 min and then precipitated with 30 µl of 20% PEG 6000 in the presence of 2.5 M NaCl. After incubation on ice for 20 min, the DNA pellet was collected by centrifugation at 15 000 rpm for 15 min, rinsed with 70% ethanol and resuspended in 30 µl 1 x TE. Although highly purified plasmid DNA was obtained, the method differed from that described below in that it does not efficiently separate nicked circular molecules from the supercoiled ccc form of plasmid DNA.

2.2.5.3. CsCl density gradient centrifugation

The volume of the plasmid DNA solution was adjusted to 3.5 ml with 1 x TE buffer and 3.77 g CsCl (1.078 g/ml) was then added to the solution. After the CsCl was dissolved, 350 μ l of a 10 mg/ml ethidium bromide solution was added. The gradient was centrifuged for 40 h at 38 000 rpm in a Beckman SW50.1 swing-bucket rotor or for 16 h at 42 000 rpm in a Beckman VTi65 rotor at 20°C. Two bands of DNA could normally be visualised on an UV transilluminator. The top band consists of chromosomal DNA as well as linear and nicked circular plasmid DNA, while the lower band consists of supercoiled ccc plasmid DNA. The lower band was collected using a hypodermic needle attached to a syringe. Ethidium bromide was removed from the solution by repeated extraction (3 or 4 times) with an equal volume water-saturated n-butanol until both the top organic and the lower water phases were colourless. The water phase (containing the purified ccc form) was diluted by the addition of 3 volumes of 1 x TE and the DNA ethanol precipitated at -20°C. DNA pellets were rinsed with 70% ethanol, dried and resuspended in 1 ml 1 x TE.

2.2.6. Characterisation of recombinant plasmids

Plasmids were isolated from colonies which were tet^R and amp^S and were analysed by horizontal electrophoresis in a 1% agarose gel with 1 x TAE electrophoresis buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.5) using plasmid pBR322 as control. Gels were prepared as described by Ausubel *et al.* (1987), adding EtBr to a final concentration of 0.5 μ g/ μ l. Possible recombinant pBR322 clones (ccc plasmids which appeared larger than control) were further selected by dot blot hybridisation and restriction enzyme digestion with *Pst*I (Boehringer Mannheim). Reactions were performed in 15-20 μ l volumes, containing 1-2 μ g plasmid DNA, $1/_{10}$ volume of 10 x restriction buffer, 10 U *Pst*I/ μ g of DNA and was incubated at 37°C for 3 h. Digestion products were analysed by agarose gel electrophoresis and the sizes of the DNA bands estimated by comparison to standard DNA markers of known molecular weight, eg. *Hind*III-digested λ DNA (SMII) and *Hae*III-digested ϕ X174 DNA (Promega).. Terminal sequences of all presumed full-length inserts were determined, by sequencing of the pBR recombinants, using primers that flank the pBR322 *Pst*I site (described in section 2.2.8).

2.2.6.1. Preparations of radiolabelled dsDNA probes by nick translation

The AHSV-9 segment 5 *Bam*H1 fragment (1.7 kb) was purified from an agarose gel using the GeneCleanTM procedure (section 2.2.7.2). The fragment was labelled radioactively by nick translation (Rigby *et al.*, 1977) which utilises the enzyme DNaseI to produce nicks in the DNA and DNA polymerase I to translate the nicks with a combination of its 5' \rightarrow 3' exonuclease and polymerase functions, incorporating radioactively labelled nucleotides. A typical nick translation reaction (Promega) contained 1 μ g linearised DNA, 0.02 mM each of dATP, dGTP and dTTP, 1 μ Ci/ μ l α -³²P-dCTP (>400 Ci/mmol; Amersham), nick translation buffer (50 mM Tris pH 7.2, 10 mM MgSO₄, 0.1 mM DTT), 1 U DNA polymerase I and 0.1 ng DNaseI. The incubation was at 15°C for 60 min and the reaction was terminated by the addition of EDTA, pH8.0 to a final concentration of 25 mM. The labelled probe was separated from the unincorporated nucleotides by chromatography on a Sephadex G-75 column. A column containing pre-swollen Sephadex beads was prepared using a 5 ml Pasteur pipette with a glass bead and equilibrated with 1 x TE buffer containing 0.5% SDS. Sample volumes were adjusted to 100 μ l with ddH₂O prior to the addition to the column after which the column was rinsed with 12 x 100 μ l buffer (1 x TE, 0.5% SDS) with simultaneous collection of fractions from the bottom of the column. Fractions were counted in a liquid scintillation counter (Beckman, LS 3801) and peak fractions were pooled.

2.2.6.2. Dot-blot hybridisation for the identification of recombinant pBR clones

Recombinant pBR plasmids were diluted to 100 μ l in 1 x TE buffer and denatured by adding 5 μ l of a denaturing buffer (0.5 M NaOH, 25 mM EDTA). Denatured DNA was spotted onto Hybond™ N⁺ nylon membranes (Amersham), pre-wetted in ddH₂O and equilibrated in 10 x SSC buffer (1.5 M NaCl, 0.15 M Na.citrate, pH 7.0), using a dot-blot apparatus coupled to a vacuum pump. Before and after spotting the DNA samples, each well was rinsed with 100 μ l 20 x SSC under vacuum. Recombinant plasmid pSPT-AH9NS1 (Section 2.2.3) was included as positive hybridisation control and pBR322 as negative control.

The adsorbed DNA was fixed to the membranes by placing the nylon filters on an UV transilluminator for 5 min on each side. The membranes with fixed DNA were prehybridised in hybridisation buffer containing 5 x SSPE (0.75 M NaCl, 200 mM NaH₂PO₄·2H₂O, 20 mM EDTA pH 7.4), 50% deionised formamide, 0.1% fat-free milk powder, 0.2% SDS at 42°C for 30 min. The labelled probes were denatured by the addition of $1/_{10}$ volume of 1 N NaOH and boiling for 5 min. The probe was placed on ice and neutralised by the addition of $1/_{10}$ volume of 1 N HCL before being added to the membrane in hybridisation buffer and incubated for 16 h at 42°C. The membrane was washed twice for 10 min in 2 x SSC at RT with mild agitation, once in 2 x SSC, 0.5% SDS at 37°C for 20 min and once in 2 x SSC, 0.5% SDS at 65°C for 15 min. Autoradiography was carried out by exposing the membranes overnight to Cronex MRF-31 X-ray film at -20°C.

2.2.7. Restriction endonuclease mapping of AHSV-6 segment 5 cDNA

Restriction endonuclease mapping of the cloned AHSV-6 segment 5 cDNA (section 2.2.5) was performed by digesting the recombinant plasmid (pBRS5.2) with each of the following restriction enzymes either individually or in combination: *Bam*H1, *Bgl*II, *Eco*R1, *Eco*RV, *Cl*aI, *Hind*III, *Hae*III, *Kpn*I, *Pst*I, *Pvu*I, *Sac*I, *Sma*I, *Sal*I, *Sty*I, *Xba*I and *Xho*I. Restriction enzyme digestions of plasmid DNA were carried out in the recommended salt buffer supplied with the enzyme (Boehringer Mannheim) for 1.5 h under the conditions as described in the protocols supplied with the restriction enzymes. Following restriction of the plasmid DNA, restriction fragments were analysed in 1% (w/v) agarose gel electrophoresis in 1 x TAE (40 mM Tris-HCL, 20 mM Na.acetate, 1 mM EDTA, pH 8.5) and sized according to their migration in the gel as compared to that of standard DNA molecular weight markers. The agarose gels were stained with ethidium bromide (0.5 μ g/ml) and the DNA fragments visualised by UV fluorescence.

2.2.8. Subcloning of AHSV-6 NS1 gene

The full-length cDNA copy (pBRS5.2) was then recloned and subcloned into the pBS (Bluescript) cloning vector for sequence determination of the complete gene. The strategy for sequencing of the full-length segment 5-specific insert, involved the construction of subclones in pBS which together would span the full-length of the NS1 gene. By making use of the compiled restriction enzyme map, the subclones were constructed as follows: The 5'-terminal region of the cDNA copy (1-503) was excised from pBRS5.2 by *Pst*I and *Hind*III digestion. A subfragment corresponding to the region from nucleotide 503 to 1450 was excised by *Hind*III and *Xho*I digestion. A third subfragment corresponding to the 3' end of the cDNA (1450-1748) was generated by digestion with *Xho*I and *Pst*I. The 500 bp, 900 bp and 350 bp fragments was cloned into the *Pst*I & *Hind*III, *Hind*III & *Xho*I and *Xho*I & *Pst*I sites of the pBS cloning vector.

2.2.8.1. Vector dephosphorilation

In order to suppress recircularisation of linearised vectors, the digested vector DNA was dephosphorylated as described by Davis *et al.* (1986). The restriction enzyme was inactivated by heating the reaction to 65°C for 10 min. Linearised vector DNA (5 µg) was incubated at 37°C for 30 min in a 50 µl reaction volume, containing a 1:10 dilution of 10 x dephosphorylation buffer (50 mM Tris-HCL, 0.1 mM EDTA, pH 8.5), and 1 U calf intestinal alkaline phosphatase (1 U CIP/µl; Boehringer Mannheim). The vector DNA was then recovered by phenol-chloroform extraction and ethanol precipitation or by GeneClean™ II procedure out of agarose gel.

2.2.8.2. Purification of restricted DNA fragments

The restricted DNA fragments were purified either by using commercially available GeneClean™ DNA purification kit, glasswool or phenol-chloroform extraction. The GeneClean™ kit was used according to the instructions of the manufacturer for the purification of DNA fragments recovered from agarose gels. Briefly, the DNA fragment of interest was excised from the agarose gel and mixed with ca. 3 volumes of a NaI solution. The agarose was dissolved at 50°C after which 5 µl glassmilk was added to the suspension. After incubation on ice for 15 min with periodic agitation, the silica-bound DNA was pelleted by brief centrifugation and washed three times with 0.5 ml ice cold NEW wash (NaCl, Tris, EDTA, ethanol, water). The DNA was eluted from the silica at 50°C for 10 min in a final volume of 15 µl ddH₂O or 1 x TE. Alternatively, the gel slice was centrifuged through glasswool packed in an Eppendorf tube, the base of which had been pierced with a needle. The DNA was then collected by ethanol precipitation. As an alternative, the restriction enzyme-digested DNA was deproteinized by phenol-chloroform extraction as described in section 2.2.4.1.

2.2.8.3. Ligation of DNA fragments and transformation

Restricted vector and segment 5-specific fragments were ligated overnight at 15°C (or 4-6 h at 30°C) in 10 µl reaction volume which contained 1 µl of a 10 x ligation buffer (660 mM Tris-HCL, 10 mM DTT, 50 mM MgCl₂, 10 mM ATP, pH 7.5) and 1 U T4 DNA ligase (1U/µl; Boehringer Mannheim). The vector:insert molar ratios were typically in excess of approximately 1:3, as estimated by EtBr staining in an agarose gel.

Half the ligation mixture was used to transform competent *E. coli* XL1-Blue cells which were then plated onto amp and tet LB-agar plates containing X-gal substrate (50 µl of a 2% stock solution) and IPTG inducer (10 µl of 100 mM stock solution). Recombinant transformants were selected by blue/white colour selection, based on the inactivation of the *lacZ* gene. Recombinant transformants with Gal^r phenotype (which gave rise to white colonies) were selected for further characterisation and grown overnight at 37°C in 3 ml of LB-broth, supplemented with amp and tet.

2.2.9. DNA Sequencing of the cloned AHSV-6 NS1 gene

Sequencing was performed on phenol-chloroform, RNase-PEG or CsCl-purified double-stranded plasmid DNA templates using the dideoxynucleotide chain-termination method of Sanger *et al.* (1977) as adapted for dsDNA templates (Hatton and Sakaki, 1986; Riley, 1989). Reactions were carried out according to the protocols of the Sequenase™ Version II sequencing kit (United States Biochemical Corporation). The sequenase™ enzyme is a modified form of T7 DNA polymerase and its properties include high processivity, high speed, efficient incorporation of nucleotide analogues and the absence of 3'→5' exonuclease activity (Tabor & Richardson, 1987). Alternative purification methods, e.g. mini-prep

sequencing (Kraft *et al.*, 1986), glassmilk (BIO 101), or Wizard column (Promega) purification were also performed. All reactions were carried out according to the manufacturers' instructions. The concentration of the plasmid DNA was determined by measuring the absorbance at 260 nm (1 OD₂₆₀= 50 µg/ml dsDNA).

2.2.9.1. Denaturation of template DNA

Double-stranded plasmid DNA has to be denatured to a single-stranded template for sequencing. Plasmid DNA (1-2 µg) was diluted to 18 µl in ddH₂O and denatured by addition of 2 µl of freshly prepared denaturing buffer (2N NaOH; 2mM EDTA). After incubation at RT for 5 min, 3 µl 3 M Na.acetate pH 4.6, 8 µl 1 M Tris-HCL pH 7.4 was added and mixed, followed by 75 µl ice cold 100% ethanol and precipitated for 30 min at -20°C. The precipitated DNA was collected by centrifugation at 15 000 rpm for 10 min, rinsed with 200 µl ice cold 70% ethanol, vacuum-dried and resuspended in 7 µl ddH₂O.

2.2.9.2. Sequencing reactions

The universal M13/pUC sequencing primers 5'-GTTTCCCAGTCACGAC-3' and 5'-GTAAAACGACGGCCAGT-3' (Boehringer Mannheim) were used for forward and reverse priming on pUC based plasmids and pBR322-PstI (+) and (-) oligonucleotide primers (5'- GCTAGAGTAAGTAGTT-3' and 5'-AACGACGAGCGTGAC-3') for sequencing of pBR322 plasmids. In addition, two synthetic internal primers were used to sequence the full 900 bp *HindIII-XhoI* fragment.

To the denatured DNA, 1 µl (1 pmol) of the appropriate sequencing primer, 2 µl 5 x Sequenase buffer (200 mM Tris-HCL pH 7.5, 100 mM MgCl₂, 250 mM NaCl) were added in a final volume of 10 µl and incubated at 37°C for 30 min to allow annealing of the primer to take place. Extension and labelling of the DNA was obtained by adding 1µl 0.1 M DTT, 2 µl 1:5 diluted labelling mix (1.5 µM of each of dCTP, dGTP and dTTP), 0.5 µl ³⁵S dATP (1200 Ci/mmol, 10 mCi/ml, Amersham) and 2 µl SequenaseTM enzyme diluted 1:8 in dilution buffer (10 mM Tris-HCL pH 7.5, 5 mM DTT, 0.5 mg/ml bovine serum albumin), mixed and incubated at RT for 5 min. Reaction termination was accomplished by the addition of 3.5 µl of the labelled incubation mix to 2.5 µl of each of the termination mixes ddATP, ddCTP, ddGTP and ddTTP (each containing 80 µM of each dNTP, 50 mM NaCl and 8 µM of the appropriate ddNTP) prewarmed to 37°C in separate microfuge tubes. Termination reactions were incubated at 37°C for 5 min and the reactions were terminated by the addition of 4 µl stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol).

2.2.9.3. Polyacrylamide gel electrophoresis

The sequencing reactions were electrophoresed in adjacent lanes of a high-resolution, denaturing polyacrylamide gel. The gels contained 6% acrylamide (w/v), 0.3% bisacrylamide (w/v) and 7 M urea as denaturant in 1 x TBE (90 mM Tris-HCL pH 8.3, 90 mM boric acid, 20 mM EDTA) and was polymerised by the addition of TEMED and 10% ammonium persulfate. The gel was pre-electrophoresed for 30 min before loading of the samples. The samples were heated to 95°C for 3 min before being loaded on the gel, and 2.5-3 µl was loaded per lane. Electrophoresis was carried out on a BRL model S2 sequencing apparatus, connected to a LKB 2197 power supply, at a constant power of 75 W (approximately 1800 V, 45 mA) in 1 x TBE. Following electrophoresis, the gel was fixed in 10% glacial acetic acid and 10% methanol to remove the urea, dried in a vacuum gel dryer for 1h at 80°C. Dried polyacrylamide gels with radiolabelled samples were exposed to Cronex MRF31 X-ray film at room temperature for 16 h or longer.

2.2.10. Preparation of AHSV-6 NS1-encoding tailored DNA copy by polymerase chain reaction

The manipulation of the segment 5 cDNA for the purpose of expression by a recombinant baculovirus, is illustrated in Fig. 1. The cDNA copy was modified by removing the 5' and 3' terminal homopolymeric dG/dC-tails (Nel and Huismans, 1990), by means of PCR (Saiki, 1988) using the enzyme Taq polymerase, and introducing two *Bam H1* sites at the 5' and 3' ends of the NS1 gene. Two 28 mer oligonucleotide primers were synthesised to modify and tailor the ends of the cloned segment 5 cDNA:

S5F: 5'-GACGGATCCAAACATGGATAGGTTCTT-3' and

S5R: 5'-GACGGATCCGATCTAATTATGCATGAAATC-3')

The primers were designed so that most of the 5' and 3' untranslated regions were deleted, but the Kozak sequence flanking the AUG was retained. These primers incorporated BamH1 restriction recognition sites at each terminal to facilitate subsequent DNA manipulations.

A PCR mixture was set up as follow. A 99.7 μ l reaction mixture was composed containing 50 ng template DNA (pBRS5.2, the pBR322 recombinant containing the full-length NS1 gene), 100 pmol (800 ng) of each oligonucleotide primer (S5F and S5R), 10 μ l of a 10 x Taq polymerase buffer (500 mM KCl, 100 mM Tris-HCL pH 8.4, 15 mM MgCl₂, 0.1% gelatin), 0.2 mM of each of dATP, dCTP, dGTP, dTTP (1 μ l dNTP mix containing 25 mM of each dNTP) and 4 U Taq DNA polymerase (5 U/ μ l; Amersham). The reaction mixture was overlaid with three drops of liquid paraffin to prevent condensation and subjected to thermal cycling (Hybaid thermocycler). The template DNA was denatured at 94°C for 5 min after which the reaction mix was subjected to five cycles, with each cycle included a denaturation step at 94°C for 60 sec followed by annealing of the primers at 52 °C for 45 sec and DNA chain extension at 72°C for 2 min. The mix was subjected to a further 25 cycles of denaturation (94°C, 60 sec), primer annealing at 58°C for 45 sec and elongation (72°C, 2 min).

The resulting PCR product was then analysed by agarose gel electrophoresis after which it was recovered and purified by the GeneClean™ procedure (section 2.2.7.2). The identity of the purified product was confirmed by restriction with *HindIII* and *XhoI*, before being digested with *BamH1* and then cloned into the dephosphorylated (described in section 2.2.7.1) *BamH1* site of pBS. Recombinant pBS was further characterised by restriction enzyme mapping with *HindIII* and *XhoI* and a representative clone (pBS-S5PCR), containing the PCR copy of the NS1 gene in the correct orientation for transcription of the sense RNA to be directed by the T7 promotor, was selected.

To retain most of the original cDNA copy of the NS1 gene, the central ± 1 kb region of the cloned PCR copy was excised by partial digestion with *HindIII* (position 503) and complete digestion with *StyI* (position 1588) and replaced with the corresponding region of the cDNA copy following the same strategy as in section 2.2.3. Partial digestions were performed as described in Ausubel *et al.* (1988). 2-4 μ g pBS-S5PCR was diluted to 60 μ l in the recommended salt buffer and aliquotted to nine Eppendorf tubes in 1 x 12 μ l and 8 x 6 μ l quantities. 0.5 μ l *HindIII* (10 U/ μ l) was added to the 12 μ l aliquot on ice and then 6 μ l of the reaction mix was consecutively transferred to successive tubes in the series. The reactions were incubated for 1 h at 37°C and then analysed by 1% agarose gel electrophoresis. The reaction conditions yielding the greatest percentage of the desired partial digest, i.e. a DNA band corresponding in size to the 4.9 kb linear pBS-S5PCR plasmid, was calculated and the reaction was scaled up as follows: 1-2 μ g pBS-S5PCR was incubated with 0.4 units *HindIII* in the recommended salt buffer in a final volume of 18 μ l for 1 h at 37°C. The 4.9 kb DNA band representing the linear pBS-S5PCR, was excised from the gel and purified by GeneClean™ extraction. Recombinant pBS containing the complete NS1 chimeric gene was designated pBS-S5Hyb. The sequences of the 5' and 3' PCR ends of this chimeric gene were verified.

2.2.11. *In vitro* expression of the cloned genome segment 5 gene

2.2.11.1. *In vitro* transcription

The AHSV-6 NS1 chimeric gene, inserted into pBS transcription vector under the control of the T7 promoter (pBS-S5Hyb) was transcribed *in vitro* using T7 polymerase (Boehringer Mannheim). The purified DNA template was linearised by *Sall* restriction, which cuts at a unique recognition site downstream of the coding region of the S5 gene. After verifying complete linearisation by agarose gel electrophoresis, the enzyme was removed from the remaining sample by phenol-chloroform extraction, the DNA recovered by ethanol precipitation and resuspended in diethylpyrocarbonate (DEPC) treated ddH₂O. *In vitro* transcription reactions were performed in a 20 µl reaction mixture containing 1 µg of the linearised DNA template, 2 µl of a 10x transcription buffer (0.4 M Tris-HCl pH 8.0, 60 mM MgCl₂, 100 mM DTT, 20 mM spermidine), 30 U of human placental RNase inhibitor (Amersham), 3 µl of a rNTP mixture (2.5 mM of each rNTP), 40 U T7 RNA polymerase. The reaction was incubated for 90 min at 37°C. Samples were analysed on a 0.8% agarose gel using RNase free electrophoretic equipment.

Similarly, the AHSV-9 NS1 clones (pSPT-2.4PCR and pSPT-2.4Hyb; section 2.2.3) were linearised by *EcoR*I digestion and then transcribed. *In vitro* transcription of recombinant pSPT clones was performed in a 50 µl reaction mixture containing 2-3 µg linearised plasmid DNA, 20 U T3 RNA polymerase, 10 µl 5x transcription buffer, 10 µl of a rNTP mixture (2.5 mM of each rNTP), 5 µl 100 mM DTT and 30 U human placental ribonuclease inhibitor. Each reaction was incubated at 37°C for 90 min and aliquots were analysed as above.

2.2.11.2. *In vitro* translation

The *in vitro* synthesised mRNA transcripts were translated *in vitro* using nuclease treated rabbit reticulocyte lysates (Amersham) according to the manufacturer's instructions. The reaction mixture (50 µl) contained approximately 1 µg of *in vitro* synthesised AHSV-6 S5 mRNA, 4 µl of a 12.5x translation mixture lacking methionine, 2 µl 2.5 M potassium acetate, 1 µl 25 mM magnesium acetate, 20 µl rabbit reticulocyte lysate and 15 µCi of [³⁵S]-methionine. The reaction was incubated for 90-120 min at 30°C. Samples of the reaction mixture were resolved by SDS-PAGE and the translated products visualised by autoradiography. As a control, [³⁵S]-labelled proteins from AHSV-3-infected CER cells were included in the analyses.

Preparations containing 0.8-1.2 µg of purified AHSV-9 S5-specific mRNA transcripts were incubated in 10 mM MMOH at RT for 15 min for denaturation or alternatively incubated for 5 min at 85°C. Denatured mRNA transcripts were translated *in vitro* in a nuclease treated rabbit reticulocyte lysate system (Amersham). Each translation reaction (25 µl) contained 1-2 µg mRNA, 2.0 µl ³⁵S-methionine and 16 µl rabbit reticulocyte lysate and was incubated at 30°C for 90 min prior to SDS-PAGE gel electrophoresis.

2.2.11.3. SDS-polyacrylamide gel electrophoresis (PAGE)

Protein samples were treated with an equal volume of 2x protein solvent buffer (125 mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol), heated to 95°C for 5 min, sonicated and stored at -20°C until used. The samples were then separated by 12% SDS-PAGE as described by Laemmli (1970). The stacking gels contained 5% acrylamide in 1x stacking buffer (125 mM Tris-HCl pH 6.8 and 0.1% SDS) and the resolving gels 12% acrylamide in 1x separating buffer (375 mM Tris-HCl pH 8.8 and 0.1% SDS). The gels were polymerised chemically by the addition of 0.008% (v/v) TEMED and 0.08%

(m/v) ammonium persulfate. Electrophoresis was performed with 1 x TGS electrophoresis buffer (25 mM Tris-HCL pH8.3, 192 mM glycine and 0.1% SDS) in the Mighty Small II SE 250 system (Hoefer Scientific Instruments) for 2-2.5 h at 100-120 V or alternatively in the Sturdiel SE400 vertical slab gel units (Hoefer Scientific Instruments) for 16 h at 80-90 V. Gels were stained in 0.125% Coomassie blue (Serva blue), 50% methanol and 10% acetic acid and destained in 5% methanol, 5% acetic acid at 50°C or 20% ethanol. Gels were dried on a slab gel-dryer for 1 h at 80°C under vacuum and dried gels exposed to Cronex MRF31 X-ray film for the appropriate lengths of time.

2.2.12. Expression of NS1 in insect cells with the BAC-TO-BAC baculovirus expression system

2.2.12.1. Construction of a recombinant bacmid transfer vector

pFastbac1, the donor vector in which the gene of choice is to be inserted, contains a strong polyhedrin promoter, upstream of an extensive MCS, to drive expression of the foreign gene. The baculovirus transfer plasmid, pFastbac1, was linearised with *Bam*H1, dephosphorylated and ligated to the full-length, tailored form of the segment 5 gene obtained by *Bam*H1 restriction from the pBS recombinant construct, pBS-S5Hyb. After transformation into competent *E.coli* X11-blue cells and plating out on LB agar plates containing amp, gent and tet, plasmids from a number of the colonies obtained were isolated and characterised by restriction analysis and PCR (section 2.2.10). A recombinant plasmid (pFB-S5Hyb), which contained the NS1 gene in the desired transcriptional orientation relative to the polyhedrin promoter, was selected by *Hind*III and *Xho*I restriction enzyme digestion and PCR using polyhedrin primer and the reverse primer S5R.

2.2.12.2. Transposition and isolation of recombinant bacmid DNA

E.coli DH10BAC™ cells, containing the bacmid genome (baculovirus shuttle vector) as well as a helper plasmid, was transformed with the recombinant transfer plasmid pFB-S5Hyb according to the manufacturer's specifications (Life Technologies). Briefly, 100 µl of competent DH10Bac cells were mixed with 100 ng donor plasmid and incubated on ice for 30 min. The cells were heat shocked at 42°C for 45 sec, 900 µl SOC medium added and incubated for 4 h at 37°C with agitation. Recombinants were selected on LB plates containing kanamycin (50 µg/ml), gentamycin (7 µg/ml), tetracyclin (10 µg/ml), IPTG (40 µg/ml) and X-gal (300 µg/ml). The bacmid genome carries the *lac Zα* gene and when used to transform *lac* DH10BAC™ cells, produces a functional β-Galactosidase protein. Therefore, cells containing the bacmid genome produce a blue colour on plates containing X-gal and IPTG. Transposition of the *Tn7* element into the bacmid genome by the pFastbac1 plasmid results in the disruption of the *lac Zα* gene resulting in the production of white colonies on plates containing X-Gal and IPTG. The white colonies are therefore bacmid-containing colonies that have undergone successful transposition and are thus selected for isolation of recombinant bacmid DNA. Several white colonies were selected and replica plated onto fresh LB-agar plates containing antibiotics and histochemicals, in order to distinguish true whites. Once a suitable colony was found, a liquid culture for isolation of composite bacmid DNA was set up.

High molecular weight recombinant bacmid DNA was isolated by the rapid alkaline lysis method modified for the isolation of large plasmid DNA (Amemiya *et al.*, 1994). The recombinants were verified by means of PCR of the isolated bacmid DNA, using primers S5F and S5R. One of the recombinant clones were selected randomly and was designated BAC-5.2Hyb.

2.2.12.3. Transfection of Sf9 cells with the recombinant baculovirus shuttle vector

The purified recombinant bacmid DNA (BAC-S5Hyb) was transfected into *S. frugiperda* cells using cellfectin reagent™ (Life Technologies), according to the instructions of the manufacturer. This is a DNA transfection procedure which makes use of a synthetic cationic lipid that forms liposomes which interact spontaneously with DNA, fuse with tissue culture cells and facilitate the delivery of functional DNA into the cell (Felgner *et al.*, 1987; Summers & Smith, 1987). Sf9 cells were seeded in 35 mm wells (1.2×10^6 cells/well) and following attachment of the cells for 1 h at RT, were rinsed twice with Grace's medium, lacking FCS (serum proteins inhibits lipofection). A further 1 ml FCS-free medium was then added to the cells. The recombinant bacmid DNA (1 µg) was diluted with 100 µl Grace's medium without antibiotic and FCS. In a separate tube, 6 µl of the cellfectin reagent was diluted to 100 µl with Grace's medium. Just prior to transfection, the two solutions were mixed and added dropwise to the monolayers of SF9 cells. After an incubation period of at least 5 h at 28°C under humid conditions, the transfection mixtures were removed and replaced with 2 ml Grace's medium supplemented with 10% FCS. The cells were then incubated at 28°C until evidence of baculovirus-infected cells was obtained (3-4 days). The supernatant, containing the recombinant baculoviruses (BV-NS1), was harvested from transfected cells and used for plaque assays. Viral stocks were prepared from single plaques using standard virological procedures described by Summers and Smith (1987).

2.2.12.4. Virus titration and plaque purification

In order to obtain purified single plaques or to determine virus titre, a method based on the procedures described by Brown and Faulkner (1977), Possee and Howard (1987) and Kitts *et al.* (1990) was used. A dilution series of the transfection supernatant or virus stock was prepared from 1.0×10^{-1} to 1.0×10^{-9} in 1 ml medium. Sf9 cells were seeded in 35 mm diameter wells at a density of $1.2-1.5 \times 10^6$ cells/well and after adsorption for 1 h at RT, the medium was replaced with 1 ml of the virus dilutions. The virus was left to adsorb to the cells for 2 h after which the inoculum was removed and the cells gently overlaid with sterile 3% low melting agarose at 37°C diluted 1:1 in Grace's medium. The dishes were incubated at 27°C for 4 days in a humid environment. The cells were then stained with 2 ml Neutral Red (100 µg/ml in Grace's medium) for 5 h at 27°C. The liquid overlay was removed and the dishes were incubated overnight before screening the plaques. Putative recombinant white plaques were removed as an agarose plug with a sterile pasteur pipette, transferred to 1 ml Grace's medium, vortexed and stored at 4°C (approximately 10^4 pfu/plug). High titre viral stocks were prepared by infecting monolayer cultures at a MOI of 0.01 to 0.1 and harvesting the virus at 96 h p.i.

2.2.13. Radiolabelling and SDS-PAGE analysis of recombinant viral proteins

Monolayers of Sf9 cells were infected with wild type or recombinant baculovirus stocks (BV-NS1) at a multiplicity of 10-15 pfu/cell and incubated at 28°C. Cells were harvested at 72 h post-infection, washed with phosphate-buffered saline (1 x PBS) and resuspended in 10 mM Tris (pH 7.4), 1 mM EDTA, 150 mM NaCl (0.15 M STE). Equal volume 2 x protein dissociation buffer was added to the sample and the proteins analysed by 12% SDS-PAGE and the resolved proteins were stained with Coomassie brilliant blue.

For radiolabelling of viral proteins, Sf9 cells were infected as described above. After 2 h, the inoculum was replaced with Grace's medium to ensure synchronised infection, and incubated at 28°C for 36 h. The medium was replaced with 500 µl methionine-free Eagle's medium and incubated at 28°C for 1 h to

deplete intracellular pools of methionine. The met-free medium was replaced with 500 μ l fresh methionine-free Eagle's medium supplemented with 15 μ Ci/ml [35 S]-methionine, followed by a labelling period of 3 h with gentle agitation. Cells were harvested and treated as above prior to loading on a SDS-PAGE gel.

For the preparation of radiolabelled AHSV-3 viral proteins, 75 cm^3 monolayers of BHK or CER cells grown in Eagle's medium containing 6% FCS were rinsed with serum-free Eagle's medium and infected with 2-3 ml AHSV-3 inoculum with high titre. After 1 h at 37°C, 5 ml additional serum-free Eagle's medium was added and incubated overnight at 32°C. At 16-18 h pi, the virus was removed and replaced with 3.0 ml methionine-free Eagle's medium. After 1 h at 32°C, the medium was replaced with 3 ml fresh methionine-free Eagle's medium to which approximately 30 μ Ci/ml [35 S]-methionine was added. After a 3 h incubation period at 37°C, the cells were washed with PBS and then resuspended in 300 μ l lysis buffer (50 mM Tris-HCl (pH 7.4), 20 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.1% SDS). MOCK infected Sf9 and BHK or CER cells were labelled in the same manner for use as controls.

2.2.14. Purification of NS1 tubules

Monolayers of Sf9 cells were infected with the recombinant baculovirus at a MOI of 5 pfu/cell and the infected cells were harvested 3 or 4 days post-infection. The cells were harvested, washed with 1 x PBS, and collected cells were resuspended in 0.15 M STE buffer and the cell membranes mechanically disrupted with a dounce homogeniser. The nuclei was then removed from the cytoplasmic fraction by low speed centrifugation (2000 rpm for 5 min) and the pellet washed once with 0.15 M STE and the supernatant was added to the cytoplasmic fraction. The cytoplasmic extract was loaded onto a 20 to 50% (W/V) sucrose gradient in 0.15 M STE (pH 7.4) buffer. Centrifugation was carried out for 3 h at 40 000 rpm using a SW50.1 rotor. The gradient was fractionated and a portion of each fraction was subjected to gel electrophoresis, following TCA precipitation of the proteins. The fractions containing predominantly the NS1 protein were pooled after which protein complexes were collected by centrifugation for 2 h at 40 000 rpm. The pellet was resuspended in 0.15M STE buffer.

2.2.15. Electron microscopy and biophysical analysis of tubule morphology

The purified NS1 tubules were adsorbed onto carbon-coated copper 400-mesh electron microscopy grids for 1 min, washed with water, and negatively stained with 2% potassium-phosphotungstic acid (pH 6.4 and 7.0) or with 2% (w/v) uranyl acetate. Alternatively, purified NS1 was adsorbed for 10 min on a grid, washed with 1 x PBS and incubated for 30 min in different dilutions of guinea pig anti-AHSV-6 antiserum. The grids were washed with ddH₂O and negatively stained with 2% (w/v) uranyl acetate and observed in a Hitachi H-600 electron microscope at 75 kV. Freeze-dried specimens were prepared on 5 mm diameter carbon discs. Samples of the tubule suspension (5 μ l) were applied to the surface of the discs which were blotted with the filter paper for 1-6 s and then rapidly plunged into liquid propane cooled by liquid nitrogen at -170°C. The frozen specimens were then freeze dried at -80°C and coated with chromium in a Gatan 681 ion beam coater. The discs were observed in a Jeol 6000 field emission SEM. Monolayers of Sf9 cells infected with the NS1 recombinant baculovirus were fixed with 3% glutaraldehyde 2 days post-infection. Thin sections were prepared as described by Huismans and Els (1979) for microscopy in a Hitachi H-600.

Aliquots of purified NS1 tubules were pelleted for 20 min at 16 000 rpm and the pellets were resuspended in various test solutions for 30 min. Following centrifugation for 5 min at 2000 rpm, the pellets and supernatants were analysed for the presence of NS1 protein by SDS-PAGE. The treated tubules (as described in the results) were also examined by means of electron microscopy after negative staining with 2% uranyl acetate.

2.3. RESULTS

When this study was initiated a cDNA copy of the NS1 gene of AHSV-9 had previously been cloned and the sequence determined. The nucleotide sequence of the cloned gene revealed that it contained the conserved specie specific 5' and 3' sequences present in AHSV and an ORF encoding 548 amino acids, indicative of a full-length copy of the dsRNA genome segment 5 (L. Nel, unpublished data). However, expression of the gene using a recombinant baculovirus failed to produce a protein of the expected size (results not shown).

The cloned AHSV-9 NS1 gene was then further characterised by *in vitro* expression to determine if the ORF had been retained during the PCR manipulations of the NS1 gene. Both a PCR tailored copy and a PCR-cDNA chimeric NS1 gene were cloned into the pSPT-19 *in vitro* expression vector in the correct orientation for transcription of mRNA, under the control of the T7 promoter located upstream from the start codon of the gene. mRNA transcripts, synthesised using T7 RNA polymerase (section 2.2.11.1), were translated *in vitro* before and after denaturation (section 2.2.11.2). The [³⁵S]methionine-labelled translation products were identified by autoradiography following SDS-PAGE. A prominent protein product of approximately 42 kDa was repeatedly found, expressing both the PCR and chimeric genes, with and without denaturation of the mRNA transcripts (*Figure 2.1*). This 42 kDa protein may represent a truncated form of the NS1 protein due to premature translation termination. The

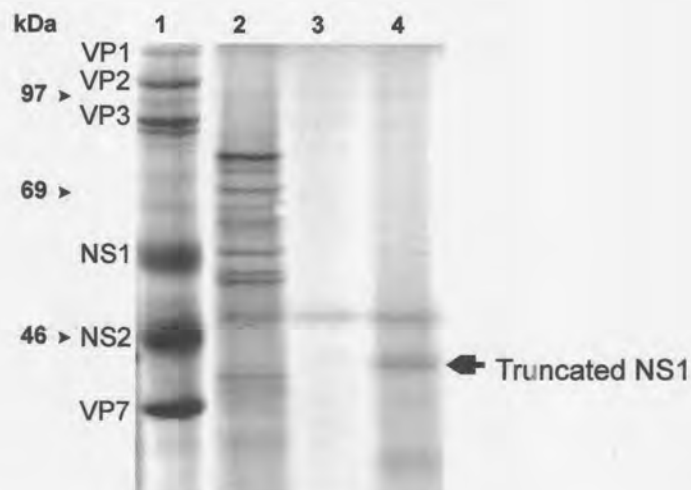


Figure 2.1: Autoradiograph of the ³⁵S-methionine labelled *in vitro* translation product of AHSV-9 NS1 gene separated by SDS-PAGE. Lane 1 represents cell lysates of CER cells infected with AHSV-3. Lanes 2 and 3 represent rabbit reticulocyte translation products in the presence and absence of any input mRNA (provided by the manufacturer) as positive and negative controls, respectively. Lane 4 contains the translation products of mRNA synthesised from the NS1 gene. The position of the truncated translation product is indicated.

truncated protein does not appear to be the result of secondary structures in the mRNA or shortened mRNA, but may be a result of an insertion or deletion mutation, which resulted in a shift in the reading frame and premature translation termination.

Since the existent copy of the NS1 gene resulted in the expression of a truncated protein, it was decided to clone a full-length NS1 gene from a pool of AHSV-6 dsRNA enriched in segments 4, 5 and 6.

2.3.1. Cloning and characterisation of AHSV-6 NS1 gene

AHSV serotype 6 dsRNA was isolated from infected CER cells by Grant Napier using the phenol-SDS method (Huisman & Bremer, 1981) and contaminating ssRNA and tRNA removed by LiCl precipitation and sucrose gradient fractionation, respectively. Double-stranded cDNA was prepared from a pool of medium-sized dsRNA fragments, purified by two successive sucrose gradient fractionation steps, using the methods described by Huisman and Cloete (1987). Since the separation of segment 5-specific cDNA from segments 4 and 6 was impossible, the pool of synthesised cDNA of AHSV-6 medium-sized dsRNA segments, including segment 5, was cloned into the *Pst*I site of pBR322 by the dG/dC-tailing cloning method (outlined in *Figure 2.2A*). Plasmid DNA was extracted from the 14 ampicillin-sensitive and tetracycline-resistant recombinants (pBR-S5.1 - pBR-S5.14). All plasmids that appeared to contain inserts after sizing were characterised further. The identity of clones with possible NS1 inserts were verified by means of dot blot hybridisation, using a radiolabelled AHSV-9 NS1 gene (pSPT-2.4PCR) as a probe (*Figure 2.3A*). The size of the inserts was estimated by excision with *Pst*I and comparison of their mobilities to DNA molecular size markers on an agarose gel (*Figure 2.3B*). Of the recombinants obtained, 43% proved to be NS1-specific, for they subsequently hybridised with the probe. In four of the clones the inserts were approximately 1.7 kb in size and was assumed therefore to be full-length NS1 genes, while two other clones (p5.4 and p5.9) contained smaller inserts (1.2 and 1.5 kb) and were probably partial NS1 clones. The other inserts were either VP4 or VP5-specific. Two representative full-length clones of the NS1 gene were selected. The sizes of the inserts in both recombinant plasmids were found to be approximately 1.7 kb and the clones were sequenced in triplicate in both directions. The nucleotide sequences of the 5' and 3' terminal ends revealed the presence of the conserved 5' and 3' hexanucleotides, which proved that the inserts represented full-length clones of the AHSV-6 NS1 gene. The two AHSV-6 segment 5-specific cDNA clones were designated pBR-S5.1 and pBR-S5.2.

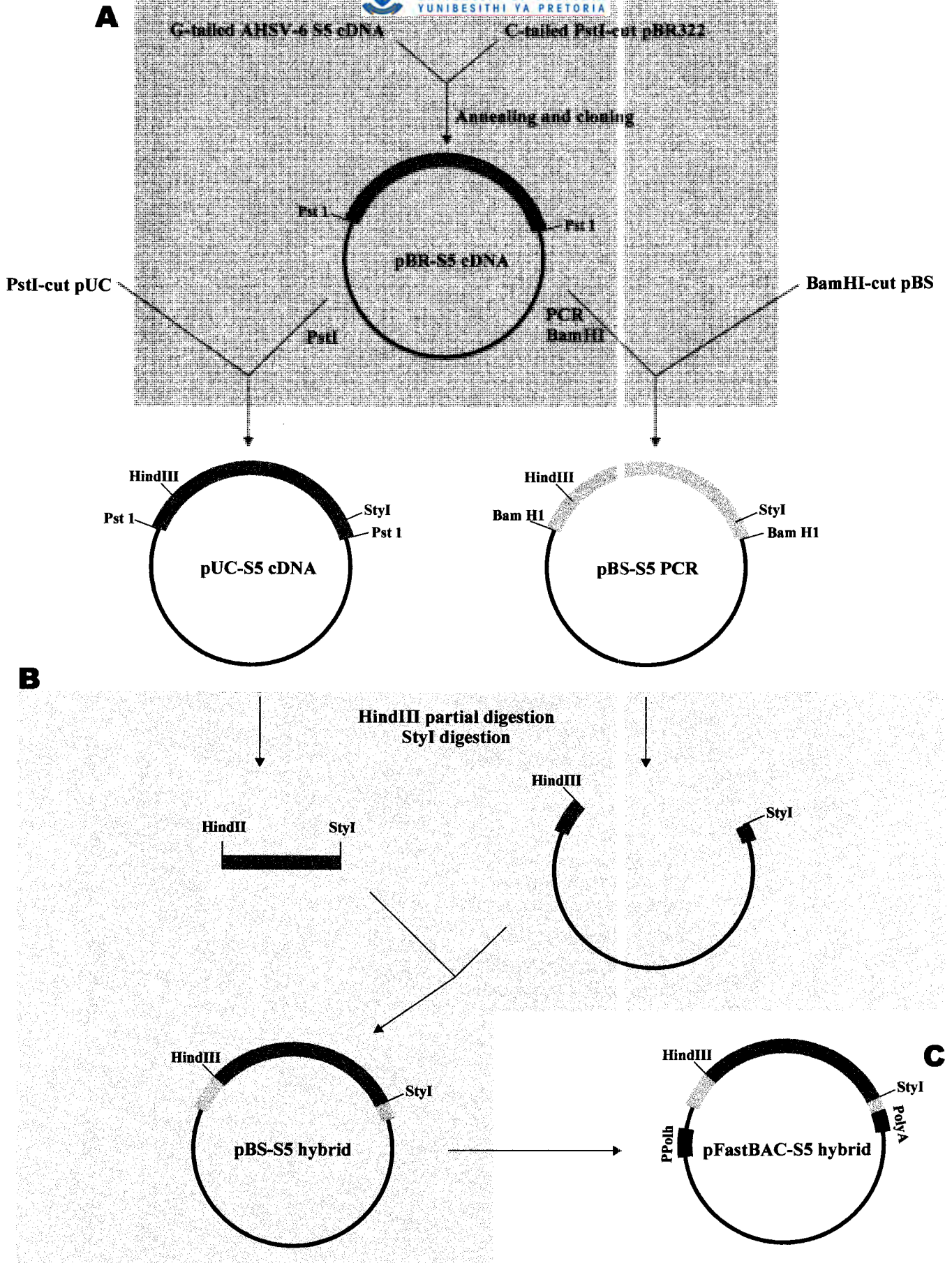


Figure 2.2: Cloning strategy for expression of NS1. A full-length cDNA copy of AHSV-6 NS1 gene was cloned by dG/dC-tailing into pBR322 (A). The cDNA copy of the NS1 gene was cloned into pUC18 for sequencing purposes. The homopolymeric tails were removed by means of PCR and a chimeric gene constructed between the PCR and the original cDNA copy of the NS1 gene (B). Finally a recombinant baculovirus transfer vector, containing the NS1 chimeric gene, was constructed (C). See text for details.

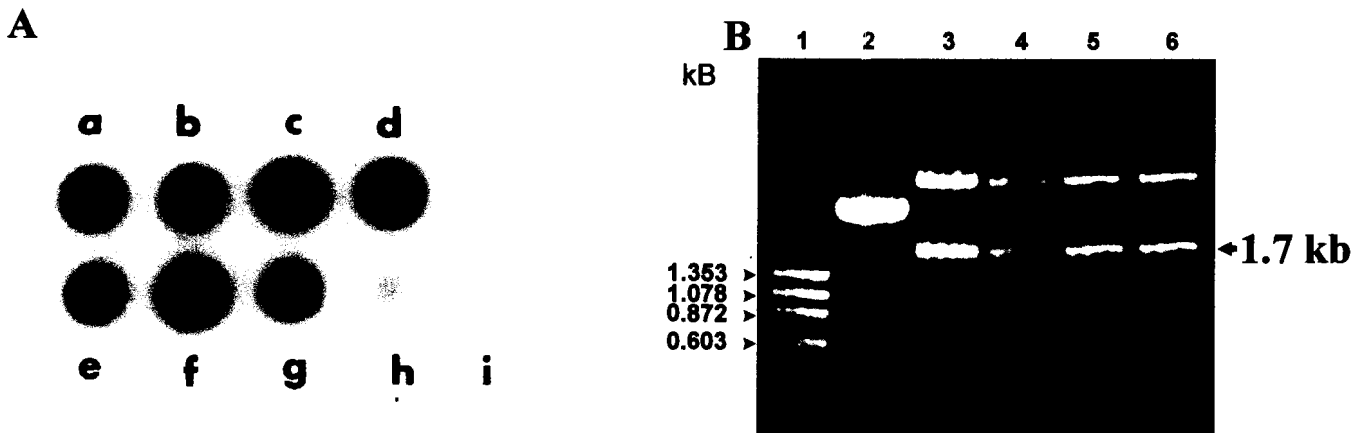


Figure 2.3: (A) An autoradiograph representing dot blot hybridisation of a ^{32}P -labelled AHSV-9 segment 5-specific probe to the recombinant pBR322 plasmids to confirm the identity of the inserts. Controls were plasmid pSPT-AH9NS1 (d), wild type pBR322 (h) and pBS-AH9VP5 (i). The recombinant pBR clones represented in a-c and e-g were p5.1, p5.2, p5.4, p5.7, p5.8 and p5.9. (B) Agarose gel electrophoretic analysis of recombinant plasmids, derived by cloning the cDNA copy of AHSV-6 segment 5 by means of dG/dC-tailing into the *Pst*I site of pBR322. The recombinant plasmids p5.1, p5.2, p5.7 and p5.8 after restriction with *Pst*I (lanes 3 to 6). The 1.7 kb insert is indicated by an arrow. Lane 1 contains the molecular weight marker and the sizes are indicated to the left of the figures. Lane 2 contains linearised pBR322.

2.3.2. Restriction enzyme mapping and subcloning of AHSV-6 NS1 gene

The strategy for determining the complete sequence of the AHSV-6 NS1 gene necessitated the construction of a series of overlapping restriction endonuclease subclones in pBS. Limited restriction maps of the clones were prepared by individual or combined digestion with various restriction enzymes, described in materials and methods. The restriction enzyme analysis revealed the presence of a *Hind*III site approximately 500 bp from the 5' end, *Xho*I, *Sma*I and *Sty*I sites 300 bp, 40 bp and 150 bp from the 3' end respectively. The compiled restriction enzyme map of the NS1 gene is shown in *Figure 2.4*. The restriction sites were used to prepare the sub-fragments from the full-length NS1 genes (S5.1 and S5.2) which were subcloned into pBS.

Recombinants of interest were purified using different DNA purification methods for sequencing (section 2.2.5). The best sequencing results were obtained with RNA-free CsCl-purified ccc plasmid DNA, although phenol-chloroform purified plasmids also gave consistent good results and were used for most of the sequencing.

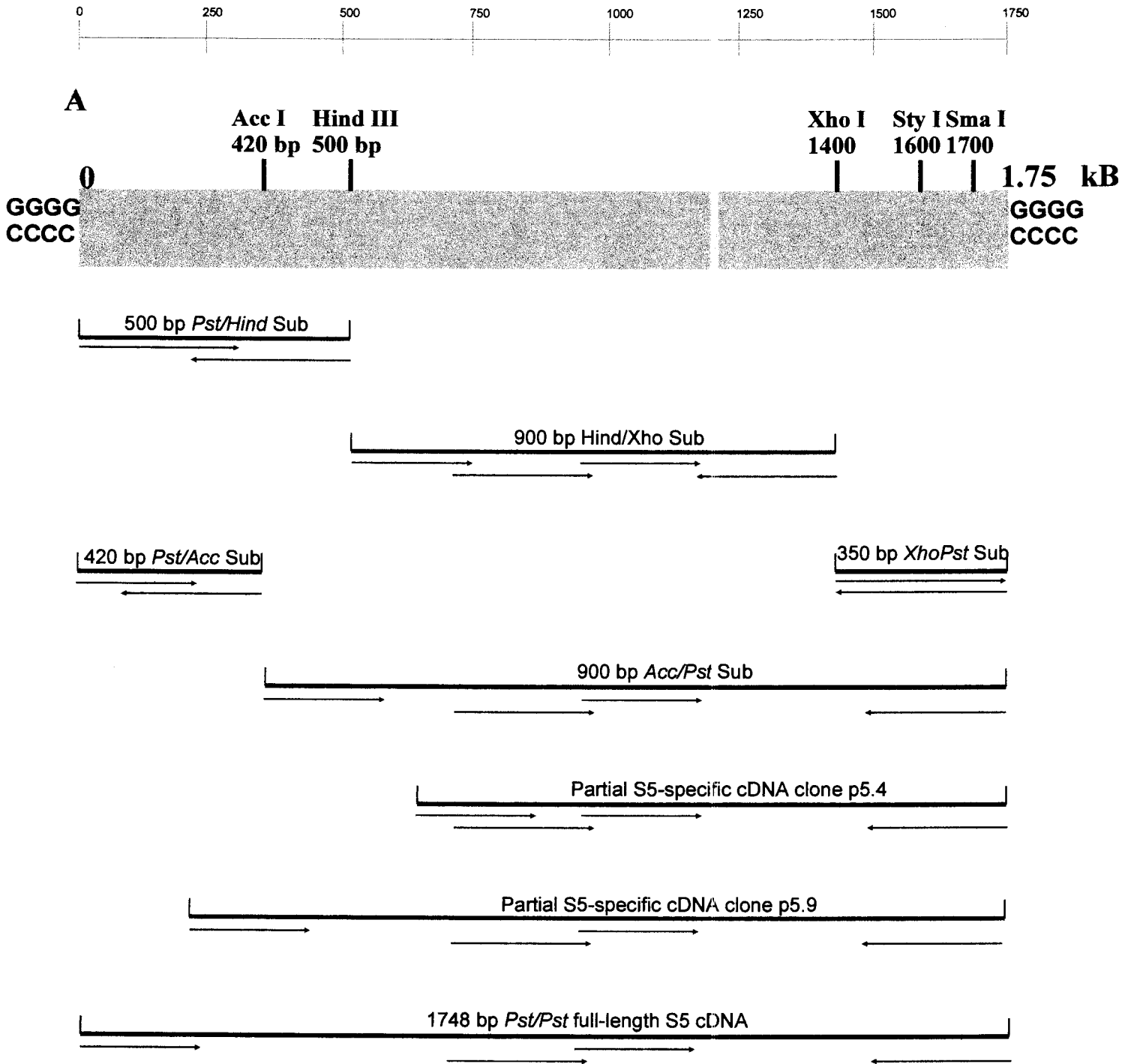


Figure 2.4: NS1 subclones prepared from NS1-specific cDNA clones p5.1 and p5.2 for sequencing, positioned relative to the full-length gene. The subclones are indicated below the NS1 gene, with the position of the restriction sites indicated on the full-length gene as approximate positions in bp from the 5' end of the gene, estimated from gel sizing analysis. Arrows indicate the distance each subclone was sequenced, using M13 primers. Internal NS1-specific primers were used to reach the center of the NS1 gene. Two partial NS1-specific cDNA clones (p5.4 and p5.9) were also used for sequence determination.

2.3.3. Characterisation of the AHSV-6 NS1 gene and deduced amino acid sequence

2.3.3.1. Nucleotide sequence of the AHSV-6 NS1 gene and comparison to the cognate genes of other orbiviruses

The complete nucleotide sequence of AHSV-6 segment 5 gene was determined using different restriction endonuclease subclones, derived from the full-length cDNA copy, in addition to the intact full-length and truncated, overlapping cDNA clones. *Figure 2.4* illustrates the regions of the NS1 gene represented in the different clones and the portion of each clone, which was sequenced. All templates were sequenced at least twice and in both orientations. Sequencing was carried out with the appropriate pBR322-specific primers, M13/pUC forward or reverse primers or with synthetic oligonucleotide primers based on previously determined sequences. By merging the sequences, the complete nucleotide sequence of the NS1 gene of AHSV-6 (*Figure 2.5*) was compiled using the computer program CLUSTAL W (Higgins & Sharp, 1988).

The complete NS1 gene of AHSV-6 (accession number U73658) was found to be 1748 nucleotides in length, with a calculated base composition of 29.98% A, 25.30% G, 28.27% T and 16.45% C. The longest, single open reading frame (ORF) observed contained 1645 base pairs and begins with a translation initiation codon, AUG, at positions 36 to 38. The ORF extended for 548 codons and terminated at nucleotide positions, 1680 to 1682 with an UAA codon. The gene was flanked by a 5' and a 3' non-coding region of 35 and 70 nucleotides, respectively. The first 6 nucleotides at the 5' end and the last 6 at the 3' end of the gene are identical to the conserved sequences at the termini of the species AHSV, RNA segments (5'GTT^A/T^A/T... and ...AC^A/T^TTAC3')(Van Staden *et al.*, 1991; Yu *et al.*, 1988). In addition, a near perfect inverted repeat sequence of 11 to 12 bp was identified in the 5' and 3' non-coding regions of the AHSV-6 NS1 gene (see *Figure 2.5*). The flanking sequences of the AUG codon, CAAACATGG, conformed fully to the eukaryotic consensus sequence for initiation of translation (CX^A/G^CXCAUGG) proposed by Kozak (1981, 1984 & 1987). The ATG codon is flanked by an A at position -3 and G at position +4 respectively, which is a favourable context for the initiation of protein synthesis according to Kozak's model.

In comparative analyses with analogous orbiviral genome segments (*Table 2.1*), AHSV-6 genome segment 5 was found to be very similar in composition to AHSV-4/9, BTV-10/13/17 and EHDV-2. Homology comparisons of the AHSV-6 segment 5 gene and encoded protein to the cognate genes and proteins of AHSV-4 and 9 were performed at sequence level using the CLUSTAL W and ANTHEPROT (Deléage *et al.*, 1988; Deléage *et al.*, 1989) computer

programs. The nucleotide comparison revealed a 97%, 94% and 93% conservation of nucleotides in type and position between serotypes 4 and 6, 4 and 9, 6 and 9 respectively (Table 2.2). Although the 3' non-coding region of the NS1 encoding gene of AHSV-4 was three nucleotides shorter than AHSV-6 and AHSV-9, the non-coding regions were identical among the three AHSV serotypes.

gaacctaggcggttggcaacacacaaac**ATG**GATAGGTTCTTGACTTATTTCCAGGTACGAGGAGAGAGA
 GCAAACGCTGTTCCGGCTGTTTGGAGAGATTTCCGAACAAATAGATTGCTCACATCTCAAACGAGATT
 GCTTTGTAAATGGAATATGTGCAAGACAACACTTTAAAGAATGCTGTAATATTGCTACAGATAATGGC
 TCACGCACAAATGCAGATAAACTAGTGGCTTTAGCTTTGCGAGCACTTTTAGATAGACAACTATTTG
 GACTTGTGTCATCAAAAATGCGGATTACGTTAGTCAATATGCTGATGAGCAGATGGAGGAAGAAGTT
 AATAAGCTGTATGATGTCTATCTCCAGAGCGGGACGAGAGAGGAATTTGAAGGATTTAGACAGAGG
 AATAGACCGAGTAGAGTTGTGATGGATGATAGCTGCTCAATGCTCTCATATTTTACATTCCAATGAA
 TCAAGGGAATCCAGCTCCAGTTGCCAAGCTTAGCCGATGGGGTCAATTTGGAATTTGTTACTATGAT
 AGAACAAATGTTGATGGATTGATTCCGTATGATGAGATCGGTTTAGCTCAAGCTATAGACGGCCTAA
 AGGATCTGATTGAAGGGCGATTGCCCGTTTGCCCTTATACTGGAGCGAATGGTAGAATTAATGCTGT
 TTTACATTTACCATTAGAGATGGAGGTGATTATGGCGGTGCAGGAAAATGCAACACAATTAATGCGT
 AGAGCGGCACAGGATTTCAAATTCATCACACATGCTGGATGGAGGCTATATCCAAGATTGTTGCGAC
 AACGGTTCGCGATCGAGGACGCTACGGAGGGGGTGATTTCATCATGTGATGCTAGGCCATTTAAGAT
 ATTATGATGAAGATACAAGTATCGTGAAGTATCGCTCCTTAACGATGGATCTTTAGATTGGAGGACT
 TGGACAATTCCTTTACATCTGATGCGGACAGCAAGGTTGGGACATCTGCAACCGGAATCAATTTAG
 TCTTTATGCATAAAAGCCTACATGTCAGGTATGCTTTATGGTTGACCTCGCTCTGCTTGACACAATCC
 CGGTGGTTGATTCAAAGTTGCCTGAACTAACAGGAGGCACAGATGTACTTTATACACGTGCGTATG
 TACATGCGGACAATCACAAAGTGCCAAATGTCAGAGATTTGATGATGAATGAAGTCTTCAGGAAGAT
 CGATGATCATTGGGTGATTCAGAAGTGCATACAACGAAGGAAGCGATTACTGTAAGTCAATTCAG
 ATTCAGAGGTGATCAGAGGTGATGGGCAGTGGGATACTCCGATGTTTCACCAATCAATGGCTCTG
 TTAACACGATTGATTGTTTATTGGTTAACGGATGTGACTGAGAGAAGTGCTATCTTTCCGGCTGACTTG
 TTTGCAATCTTCGGATGTAAGCCAACGGCTCGAGGTAGATATATTGATTGGGATGATCTTGGAAACA
 TTCATGAAGAATGTCTTGGATGGAAGAGATTTGACTGTTTTGGAAGATGAGACATGTTTTATTTTCGAT
 GATGAGGATGGCGATGTTGCATGTGCAGAGATCCAAGGTAGTGTGCGCAACTGTGTTGGAGGCCG
 CATTAGAAATACAACAGGTTGGCCAGATCGTTGAAGTACCCTTTGATTTTATGCATA**A**ttagattaaat~~taa~~
 agtttgaccgggaagtaggtaagatcatatcccctggttcacaa

Figure 2.5: The complete nucleotide sequence of the NS1-encoding segment 5 cDNA of AHSV-6. The conserved 5' and 3' hexanucleotide sequences are highlighted in dark grey and the inverted heptanucleotide repeats in light grey. The translation initiation and termination codons are indicated in bold, while the ORF is indicated in capital letters. The ORF begins at position 36 to 38 and is terminated by the TAA codon at positions 1680 to 1682.

TABLE 2.1: Comparative analysis of orbiviral NS1 gene nucleotide sequences

Orbivirus	Length (bp)	Base composition				Non-coding region (bp)		Coding region (bp)
		%A	%C	%G	%T	5'	3'	
AHSV-6	1748	30.0	16.50	25.30	28.30	36	66	1644
AHSV-9	1751	29.98	16.45	25.30	28.27	36	69	1644
BTV-10	1769	31.0	17.7	23.7	27.7	34	79	1656
EHDV-2	1806	34.1	15.2	24.7	26.0	32	118	1656

TABLE 2.2: The degree of nucleotide conservation of the NS1 gene of different AHSV serotypes and other orbiviruses. The similarities between AHSV-6 and the latter viruses are expressed as percentages of nucleotides identical in type and position.

COMPARISON	AHSV-4	AHSV-9	BTV-10	BTV-13	EHDV-2
AHSV-6	97%	93%	44%	48%	42%

The nucleotide sequence of AHSV-6 NS1 gene was subsequently compared to the cognate genes of other orbiviruses. In a comparison of the AHSV-6 NS1 gene to that of BTV-10/13 (Lee & Roy, 1987) and EHDV-2 (Nel *et al.*, 1990), homologies of 44-48% and 42% were respectively observed. The results are summarised in *Table 2.2*. A comparison of the 5' and 3' non-coding regions of the different NS1 genes revealed that the 35 nucleotide 5' non-coding region of the AHSV-6 NS1 gene is 1 and 3 nucleotides longer than that of BTV-10 and EHDV-2 and displayed a nucleotide homology of 71% and 69%, respectively. However, the 3' non-coding region of 70 nucleotides is 9 bp and 48 bp shorter than that of the cognate BTV-10 and EHDV-2 genes and displayed a nucleotide homology of 44% and 36%, respectively. Alignment of the AHSV-6, BTV-10 and EHDV-2 NS1 gene coding regions indicated a nucleotide homology of 43% and 41%, respectively.

2.3.3.2. Amino acid sequence of the AHSV-6 NS1 protein and comparison to the gene products of other orbiviruses

The amino acid sequence of AHSV-6 NS1 was deduced from the nucleotide sequence of the NS1 gene, using the ANTHEPROT computer program (Deléage *et al.*, 1988; Deléage *et al.*, 1989). The ORF of the NS1 gene is capable of encoding a polypeptide of 548 amino acids with a calculated molecular weight of 63 478 Da and a net charge of +5 at a neutral pH (*table 2.3*).

On protein level, 95%, 92% and 96% of the amino acids were identical in type and position in the comparison between AHSV serotypes 4 and 6, 4 and 9, 6 and 9 respectively (*Figure 2.6*). When amino acids of similar character were taken into consideration the homology increased to 96%, 94% and 98% in the alignments between 4 and 9, 4 and 6, 6 and 9 respectively. The N- and C-terminal regions of the cognate proteins shared the greatest levels of homology. Alignment comparisons (*Figure 2.7*) of the NS1 amino acid sequences between AHSV-6 and BTV-10/13/17, and AHSV-6 and EHDV-1/2 revealed an overall homology of 19% and 25% respectively, when only identical amino acids were taken into account. However, when both identical and similar amino acids were considered, the homology increased to 34% and 44%, respectively. The percentage homology found in each case is summarised in *Table 2.4*. In a three way best fit alignment, the aligned NS1 sequences could be divided into alternating regions of higher and lower similarity, as shown in *Figure 2.8*, and the N-terminal ends (aa 1-180) of the NS1 proteins were found to be generally more conserved than the C-terminal ends. The central region (aa 180-400) was the least conserved. Highly conserved regions of about 10 to 20 amino acids which contained a high percentage (> 70%) of identical amino acids could be identified in the three way comparison (*Figure 2.8*).

The amino acid composition of the AHSV-6 NS1 protein, together with that of the NS1 proteins of BTV and EHDV-2, is summarised in *Table 2.3*. AHSV NS1 contains 14 cysteine residues, while the BTV and EHDV counterpart contain 16 and 17 cysteine residues, respectively. The 14 cysteine residues were found to be conserved among AHSV-4, 6 and 9 and only 7 of these were conserved between AHSV-6 and BTV-10 with two more cysteine residues conserved in the immediate region. Furthermore, the three NS1 proteins contain similar amounts of tryptophan, tyrosine, serine and threonine residues. A characteristic feature of AHSV-6 NS1 is the abundance of hydrophobic residues and the low number of charged amino acids. The NS1 protein of AHSV-6, especially the C-terminal 400 amino acids, is highly hydrophobic, with a hydrophobic amino acid content of more than 43.6% and only 26.1% charged amino acids. The hydropathic plots of the three AHSV serotypes are nearly identical. The predicted hydrophilicity profile (Kyte & Doolittle, 1982) of AHSV NS1 (*Figure 2.9*) indicated that the 150 amino acids at

TABLE 2.3: Comparison of the amino acid composition, net charge and estimated size of the NS1 proteins of AHSV-6, BTV-10 and EHDV-2. The deduced protein sequences were analysed using the ATHEPROT computer package.

Amino acid residues	AHSV-6 number	BTV-10 number	EHDV-2 number
Alanine (A)	36	41	36
Arginine (R)	44	43	42
Asparagine (N)	20	20	24
Aspartic acid (D)	37	32	28
Cysteine (C)	15	16	17
Glutamine (Q)	27	26	28
Glutamic acid (E)	29	39	38
Glycine (G)	29	32	32
Histidine (H)	18	14	13
Isoleucine (I)	34	39	38
Leucine (L)	55	42	37
Lysine (K)	18	23	21
Methionine (M)	21	23	32
Phenylalanine (F)	23	26	30
Proline (P)	17	20	22
Serine (S)	21	25	17
Threonine (T)	34	23	27
Tryptophane (W)	11	13	12
Tyrosine (Y)	20	28	25
Valine (V)	39	27	32
Acidic (D+E)	66 (12%)	71 (13%)	66 (12%)
Basic (R+K)	62 (11%)	66 (12%)	63 (11%)
Aromatic (F+W+Y)	54 (10%)	67 (12%)	67 (12%)
Hydrophobic (Aromatic+I+L+M+V)	203 (37%)	198 (36%)	206 (37%)
Total amino acids	548	552	551
Net charge (pH 7.0)	+5	+7	+5
Size (Daltons)	63 478	64 446	64 580

TABLE 2.4: Amino acid similarity comparisons among different AHSV serotypes and other orbiviruses. The overall similarities between AHSV-6 and the other viruses observed, are expressed as percentages when only identical amino acids are taken into account and also when both identical and similar amino acids are considered (values in brackets).

COMPARISONS	AHSV-4	AHSV-9	BTV-10/13/17	EHDV-2
AHSV-6	95% (96%)	96% (98%)	19-28% (34-46%)	25% (44%)

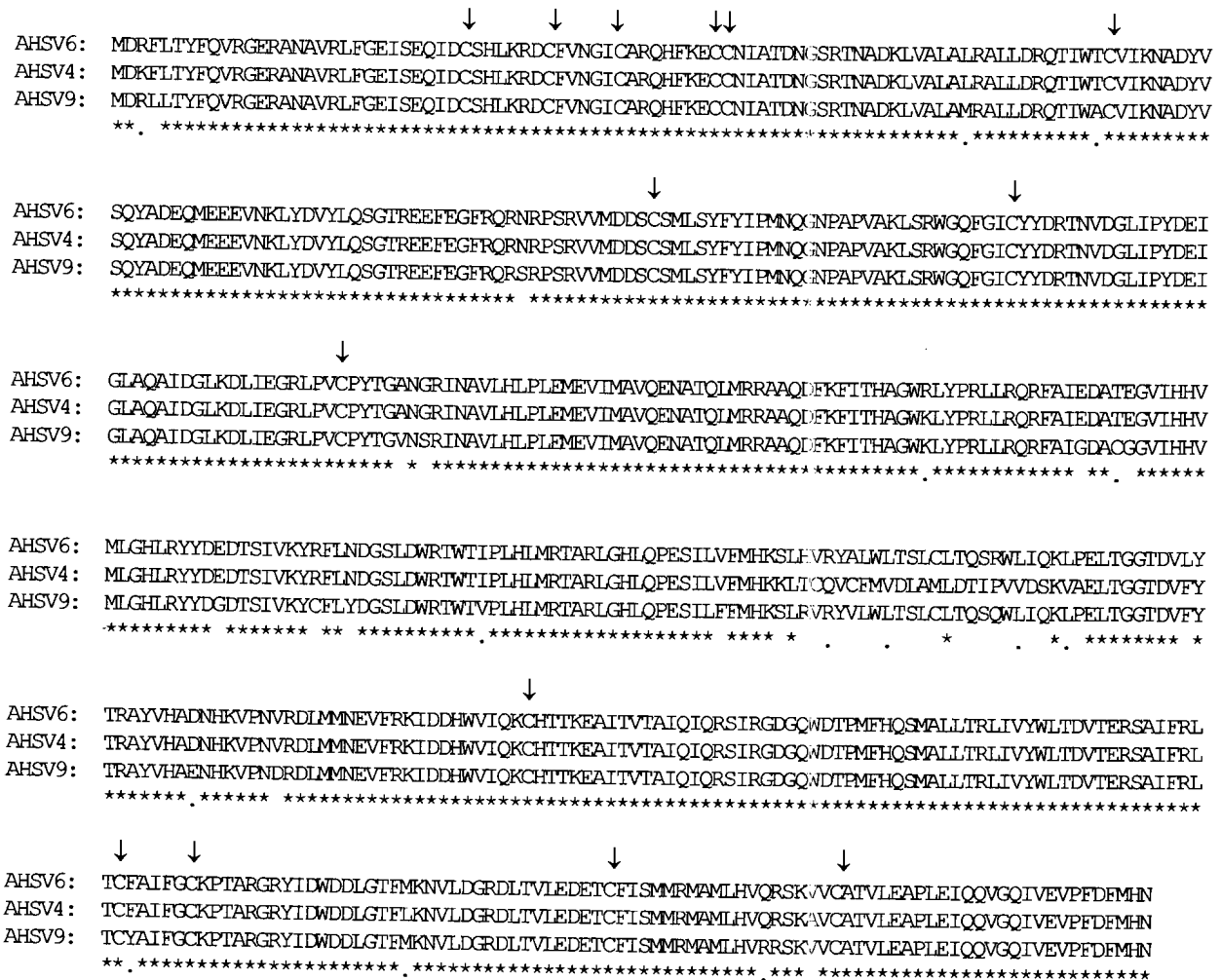


Figure 2.6: Alignment of the predicted amino acid sequences of the NS1 protein of AHSV serotypes 6, 4 and 9. Identical amino acids are indicated by asterisks (*) and similar amino acids by dots (.). In this alignment the amino acid homology is 95% and 96% and the amino acid similarity is 96% and 98% between serotypes 6 and 4 and 6 and 9, respectively. The position of 14 cysteine residues out of a total of 16 are conserved among AHSV-6, 4 and 9 (indicated by arrows).

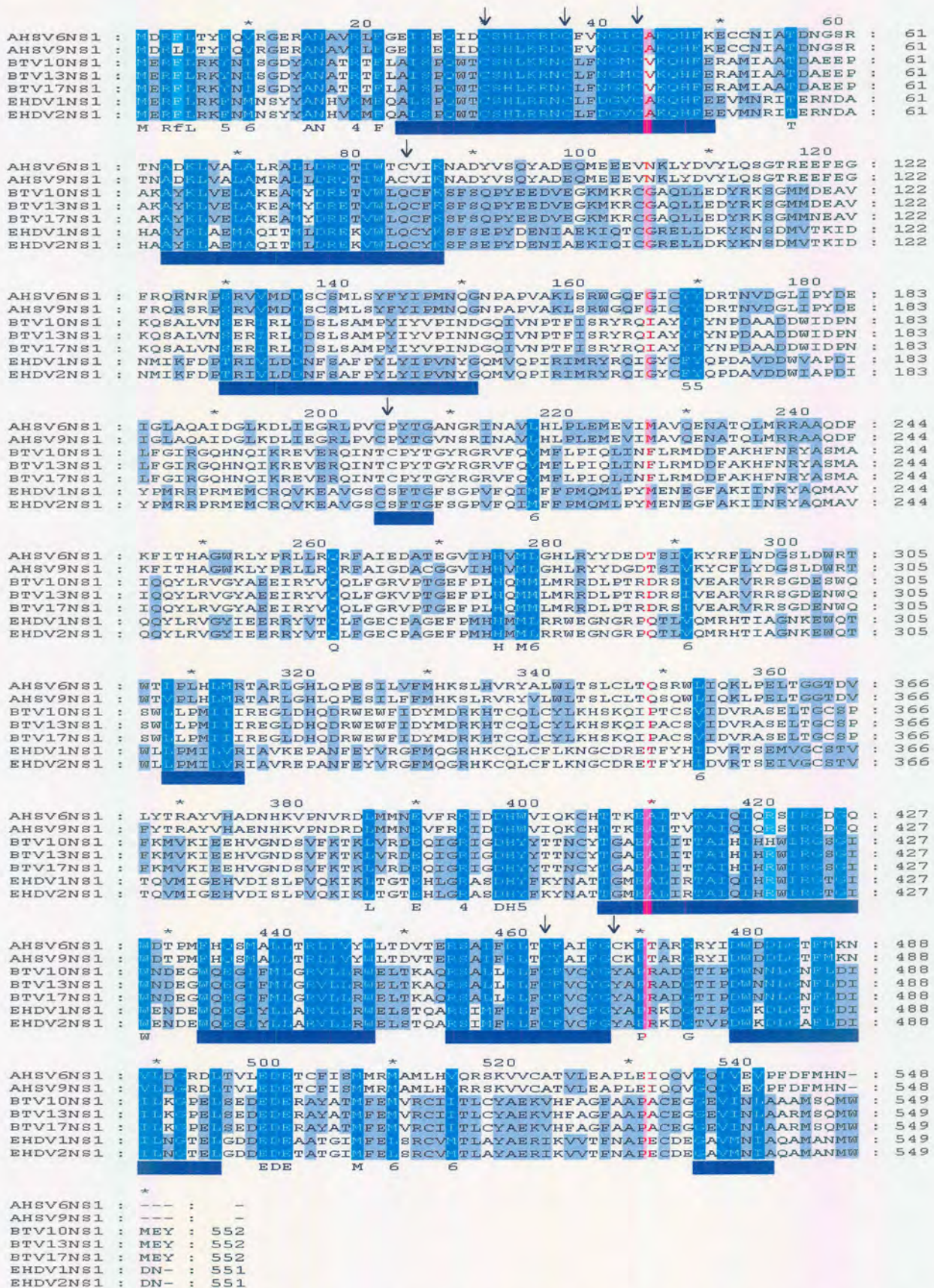


Figure 2.7: Alignment of the predicted amino acid sequences of the NS1 protein of AHSV-6, -9, BTV-10, -13, -17 and EHDV-1 and -2. Identical amino acids are highlighted in blue and similar amino acids in purple. Six cysteine residues are conserved among the three orbiviruses (arrows). The underlined areas represent highly conserved regions containing more than 70% similar amino acids.

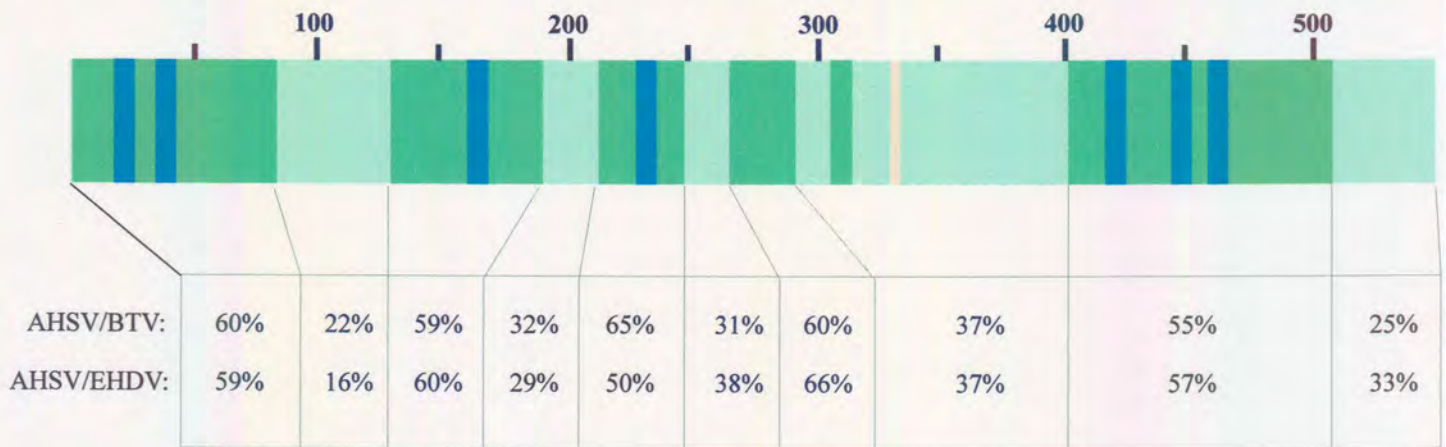


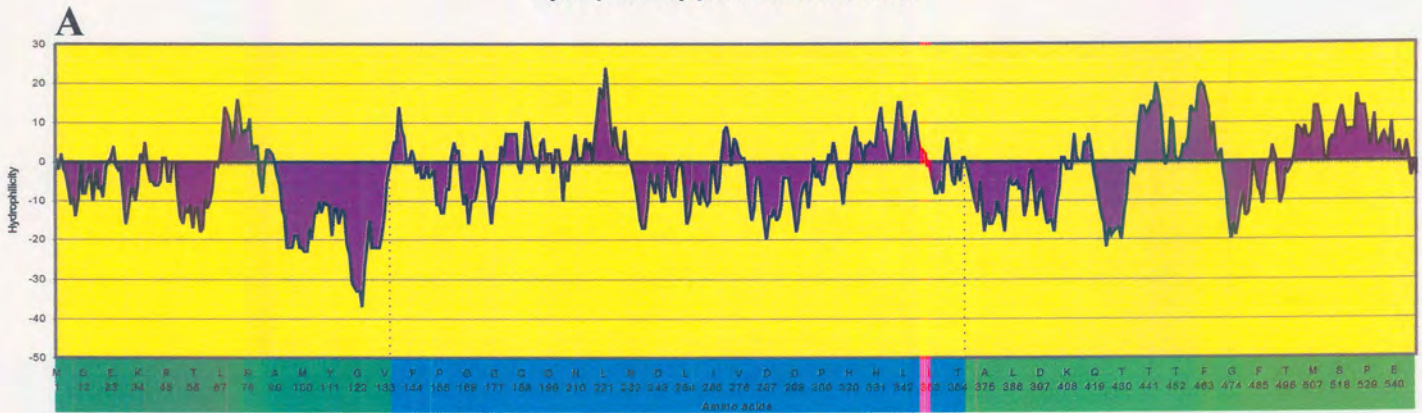
Figure 2.8: Percentage amino acid similarity in different NS1 proteins. Green represent region of >50% amino acid similarity in any two way comparison between AHSV-6, BTV-10 and EHDV-2. The dark blue areas represent regions of >70% similar amino acids in a three way comparison. The rest is regions with low similarity.

the N-terminal of the protein displayed largely hydrophilic regions with two relative small hydrophobic domains. This was followed by a large predominantly, hydrophobic domain stretching from amino acids 150 to 350. The C-terminal region was also characterised by alternating hydrophobic and hydrophilic regions, containing two relative large hydrophobic domains. The hydrophilicity profiles of the NS1 proteins of BTV-10 and EHDV-2 indicated a very high level of similarity to that of AHSV-6 NS1, particularly so in the hydrophobic regions. The hydrophobic domains were generally situated in the areas of highest amino acid similarity and their location is almost exactly conserved on the three different proteins (*Figure 2.10*).

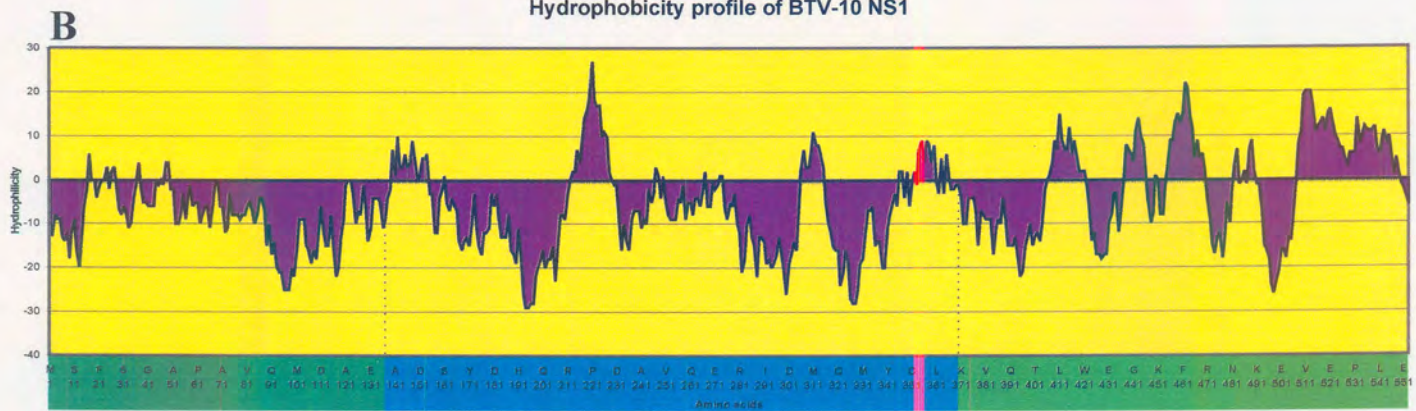
N-glycosylation motifs (Asn-X-Ser/Thr, with X any amino acid residue) as well as different kinase phosphorylation sites were found when the deduced protein sequences of AHSV-6, BTV-10 and EHDV-2 was analysed against the Prosite database (Bairoch *et al.*, 1995). The location of these sites in the NS1 proteins of the three different orbiviruses does not correlate at all. The significance of the sites is unknown. Compared to protein sequences in the SwissProt database it showed no significant homology to proteins other than NS1 of other orbiviruses.

Secondary structure prediction (*Figure 2.10*), using four different methods in ANTHEPROT, of the NS1 proteins of AHSV-6, BTV-10 and EHDV-2 indicated a number of corresponding, alternating α -helices. Five α -helices were identified in the N-terminal 150 amino acids, while the C-terminal 150 amino acids contain 7 helices. The central part of the NS1 protein contains another six α -helices. β -strands and β -turns separate these helices from each other. The hydrophilicity profile (Hopp & Woods, 1981) in combination with the antigenicity prediction (Welling *et al.*, 1989) indicated a hydrophilic region with strong antigenic properties in the C-terminal from amino acid 470 to 500 (*Figure 2.11*). The N-terminal also showed one strong antigenic site, which stretches from amino acid 55 to 77, within a hydrophobic region.

Hydrophobicity profile of AHSV-6 NS1



Hydrophobicity profile of BTV-10 NS1



Hydrophobicity profile of EHDV-2 NS1

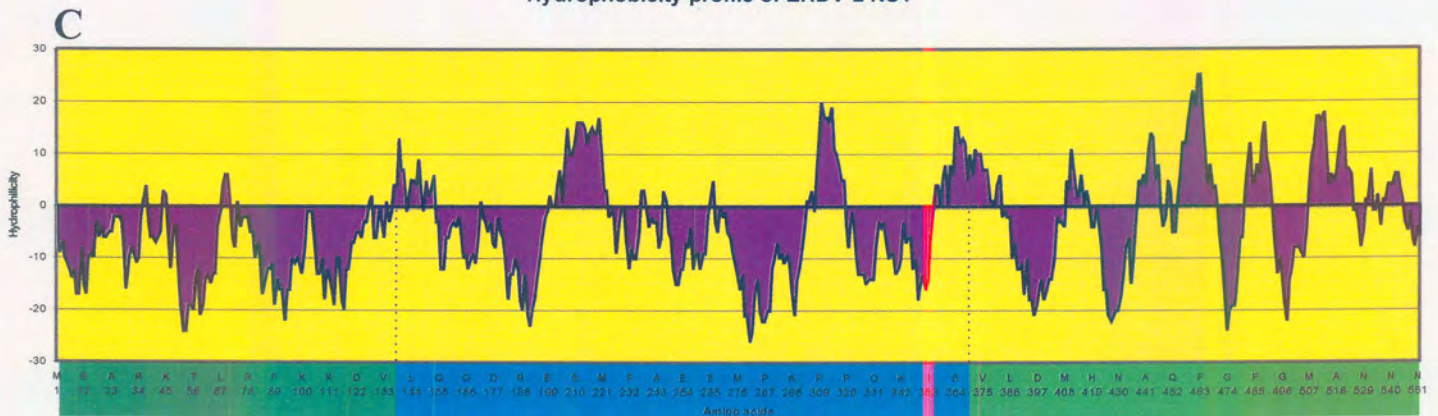


Figure 2.9: Comparisons of the hydropathicity profiles (Kyte & Doolittle, 1982) of NS1 of AHSV-6 (A), BTV-10 (B) and EHDV-2 (C), showing regions with a nett hydrophobicity (positive values) and a nett hydrophilicity (negative values) using a window size of 11. The central 250 amino acid region of the NS1 protein is highlighted in dark blue, while the N- and C-terminal regions is indicated in green.

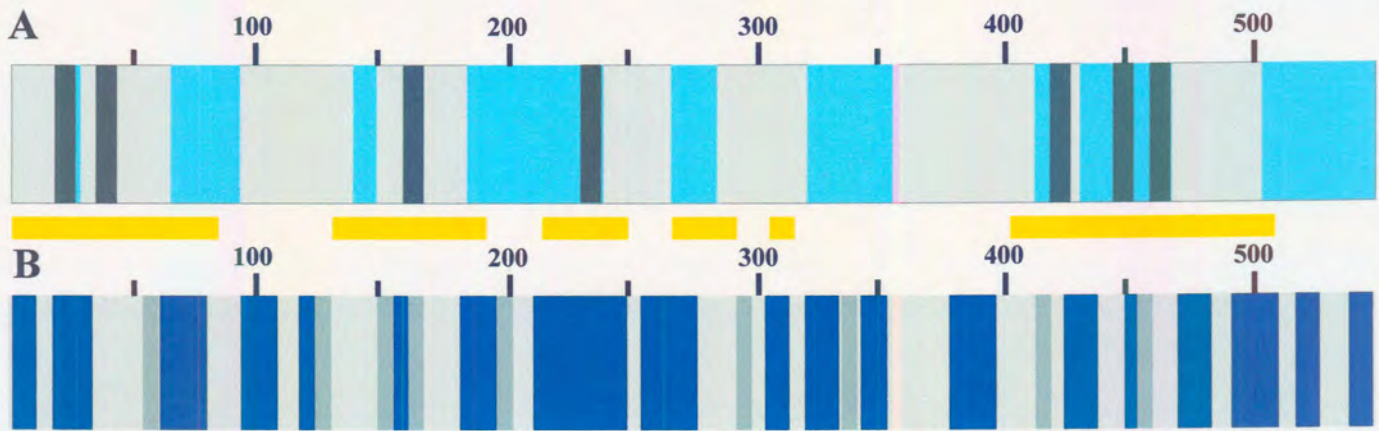


Figure 2.10: (A) Comparisons of the location of hydrophobic regions of NS1 of AHSV-6 and BTV-10. The black areas represent regions of >70% similarity in a three way comparison, while the blue areas represent conserved hydrophobic domains between AHSV and BTV. (B) Schematic representation of the secondary structure prediction of the NS1 protein of the three orbiviruses (AHSV, BTV and EHDV). The position of potentially conserved α -helices (dark blue) B-sheet (light grey) and B-turns (dark grey) among these viruses is shown relative to conserved regions (>50%; yellow boxes) as well as conserved hydrophobic regions (blue in A).

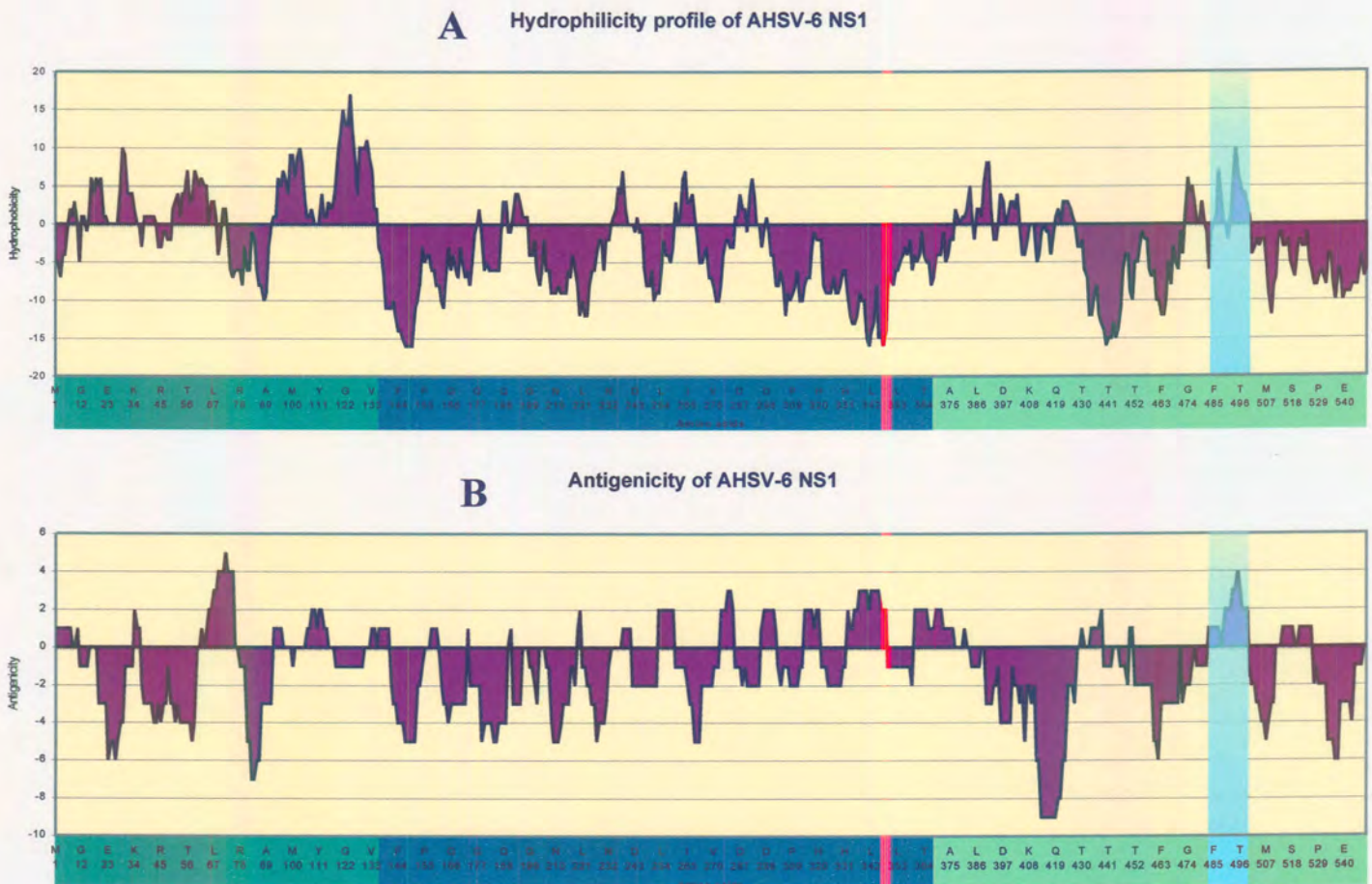


Figure 2.11: Comparisons of the hydrophilicity profile (A) (Hopp & Woods, 1982) and antigenicity profile (B) (Welling *et al.*, 1988) of AHSV-6 NS1. Areas with positive values have a nett hydrophilicity or antigenicity, while areas with negative values have a nett hydrophobicity or absence of antigenicity.

2.3.4. Modification and *In vitro* expression of the NS1 gene of AHSV-6

2.3.4.1. Modification of the NS1 gene for expression

In order to express the cloned cDNA copy of the NS1 gene, it was necessary to remove the poly-dG/dC tails flanking the 5' and 3' termini of the NS1 cDNA clone, which were present as a result of the original cloning strategy. The modification was carried out by means of a PCR method in which the oligonucleotide primers were designed to eliminate most of the 5' and 3' non-coding regions, except for the Kozak sequence flanking the AUG codon (Kozak, 1987). The primers, simultaneously incorporate flanking *Bam*H1 restriction sites in the PCR product in order to facilitate cloning into appropriate vectors (Nel & Huismans, 1991).

A PCR reaction was carried out as outlined in section 2.2.10 and the NS1 gene was successfully amplified as a discrete 1.65 kb band (*Figure 2.12A*), which corresponds to the coding sequence of the gene. The amplified product now contains the complete ORF with terminal restriction sites and is free of dC/dG homopolymer tails. Since the NS1 gene has unique *Hind*III and *Xho*I sites, 500 bp and 1400 bp from the 5' terminal end, the identity of the PCR product was confirmed by restriction with *Hind*III (*Figure 2.12A*). The PCR product was cloned into the pBS expression vector and a recombinant plasmid containing the PCR product under control of the T7 promoter was selected by restriction enzyme mapping with *Hind*III which cuts the vector once and also cuts asymmetrically in the NS1 gene. The desired recombinants yielded DNA fragments of 1.2 kb and 3.7 kb in length after digestion with *Hind*III and fragments of 350 bp and 4.6 kb after *Xho*I digestion (*Figure 2.12B*, *Figure 2.2*). Limited sequencing of the termini confirmed the full-length status of the PCR clone. Since errors could have been incorporated by *Taq* polymerase during the PCR, a chimeric NS1 gene, in which most of the central region of the PCR copy has been replaced by the original cDNA copy, was constructed in pBS as outlined in *Figure 2.2B*, instead of the conventional sequence verification. An approximately 1 kb region between position 503 and 1588 of the PCR copy (pBS-S5PCR) was excised by partial digestion with *Hind*III and complete digestion with *Sty*I (*Figure 2.13A*) and replaced with the corresponding region of the original cDNA copy (pBR-S5.2) (*Figure 2.13B*). The 500 nucleotides at the 5' end and the 90 nucleotides at the 3' end of the original PCR copy was verified by sequencing and no difference between the PCR amplified ends and the original cDNA copy of the NS1 gene was observed. Recombinant pBS (*Figure 2.13C*) containing the complete chimeric gene was designated pBS-S5Hyb.

2.3.4.2. *In vitro* expression of the NS1 gene

The NS1 chimeric gene was expressed *in vitro* from the linearised pBS-S5Hyb template. *In vitro* transcription of the NS1 chimeric gene by T7 polymerase resulted in the synthesis of a (+)-strand RNA which was translated *in vitro* in a nuclease-treated rabbit reticulocyte lysate system. The mRNA directed the synthesis of a single 63 kDa protein, which corresponded to the NS1 protein, synthesised in AHSV-3-infected CER cells (Figure 2.14). The NS1 protein was expressed in large amounts *in vitro* which indicated that deletion of the 5' non-coding region of the NS1 gene excluding the Kozak sequence, did not significantly alter the expression of the gene.

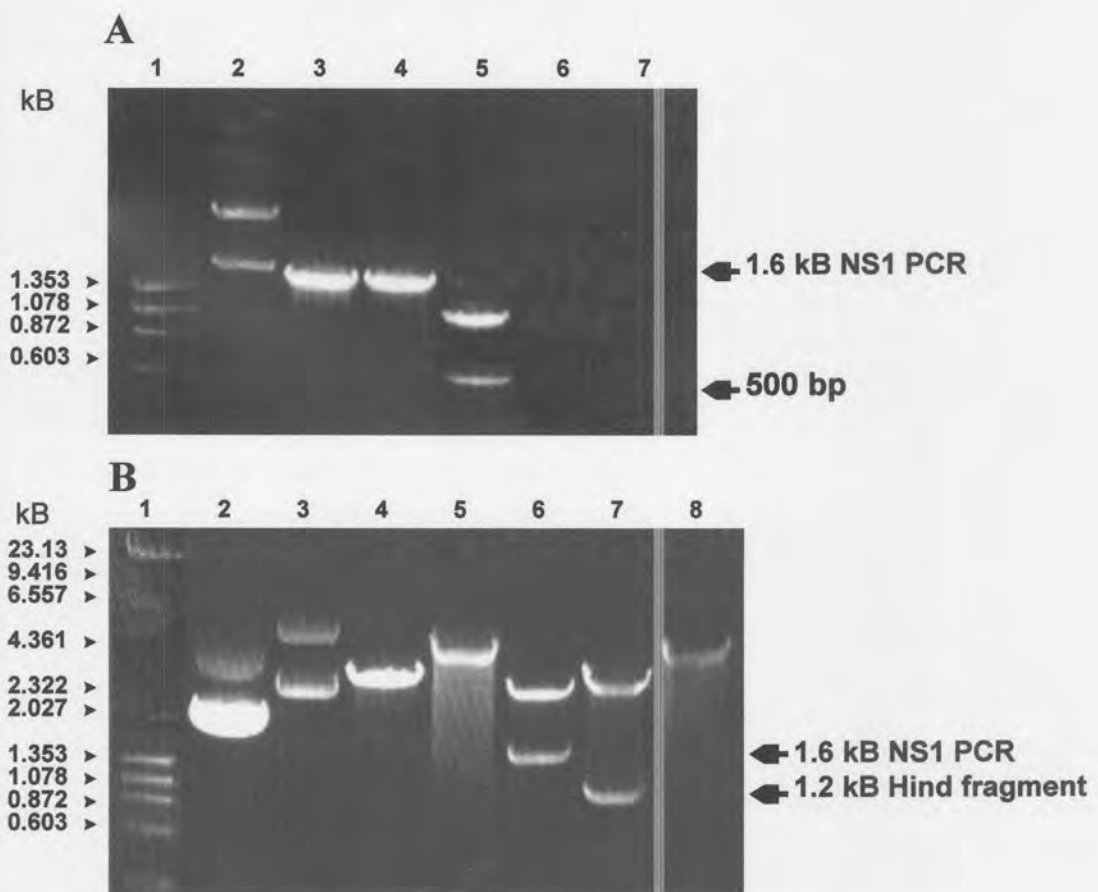


Figure 2.12: (A) Agarose gel analysis of PCR amplified fragments of AHSV-6 NS1 specific cDNA. Lane 1 represents a DNA molecular weight marker, with fragment sizes indicated in kb to the left. Lane 2 is *Pst* I digested p5.2cDNA. Lanes 3 and 4 represent the PCR amplified product of the NS1 gene. The 1.65 kB PCR fragments containing only the ORF of the NS1 gene are indicated. Lane 5 shows *Hind* III (cuts at position 502) digestion of the PCR amplified fragment, for verification. Lanes 6 and 7 are negative controls for the PCR reaction, containing no template or no primer, respectively. (B) Agarose gel analysis of the recombinant plasmid pBS-S5.2PCR (lane 3, uncut), constructed by cloning the PCR-tailored NS1 gene into the *Bam* H1 site of pBS (lane 2, uncut). pBS-S5.2PCR was linearised with *Xba* I (lane 5) or restricted with *Bam* H1 (lane 6), *Hind* III (lane 7) and *Xho* I (lane 8). See figure 2.2 for restriction enzyme map of the NS1 gene. Uncut pBS (lane 2) and linearised pBS (lane 4) were included as controls. The sizes of the DNA molecular weight markers (lane 1) are indicated to the left of the figure.

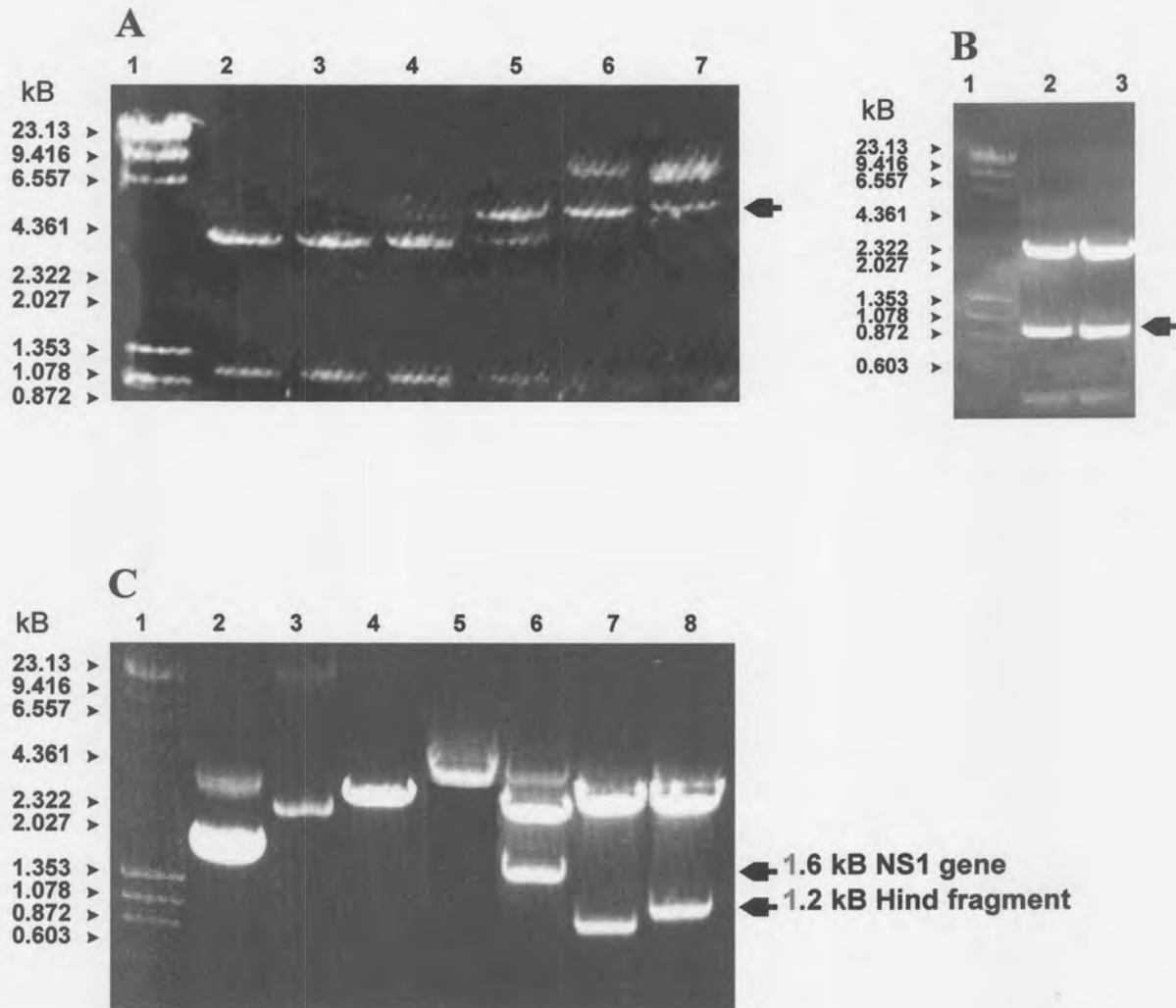


Figure 2.13: (A) An Agarose gel analysis of a partial *Hind* III digestion of the plasmid pBS-5.2PCR, through serial dilution. The sizes of fragments generated are 1.0 kb, 3.7 kb and 4.7 kb. The 4.7 kb fragment was produced after single digestion with *Hind* III and represented linear pBS-S5.2PCR (indicated by an arrow). (B) Complete *Hind* III and *Sty* I digestions of pUC-5.2cDNA (lanes 2 and 3). The 1.1 kb *Hind-Sty* fragment of the NS1 gene is indicated by an arrow. (C) Agarose gel analysis of the recombinant plasmid pBS-S5.2Hybr (lane 3, uncut), constructed by cloning 1.1 kb *Hind-Sty* cDNA derived fragment into the corresponding sites of pBS-S5.2PCR. pBS-S5.2Hybr was linearised with *Xba* I (lane 5) or restricted with *Bam* H1 (lane 6), *Hind* III (lane 7) and simultaneously with *Xho* I/*Sa* I (lane 8). See figure 2.2 for RE map of NS1 gene. The sizes of the DNA molecular weight markers (lane 1) are indicated to the left of the figure. Lanes 2 and 4 represent uncut and linearised pBS as controls.

The *in vitro* synthesised NS1 polypeptide was concentrated by centrifugation through a 40% sucrose cushion. Electron microscopic analysis of negatively stained samples revealed the presence of short tubular structures (result not shown) with a diameter of approximately 22 nm, which is similar to that of authentic AHSV tubules (Huisman & Els, 1979). The structure of the *in vitro* synthesised tubules appears to be less homogenous than that of authentic AHSV NS1 tubules described by Huisman and Els (1979). After verifying expression, the recombinant NS1 protein was further characterised by *in vivo* synthesis in insect cells, using a NS1 recombinant baculovirus.

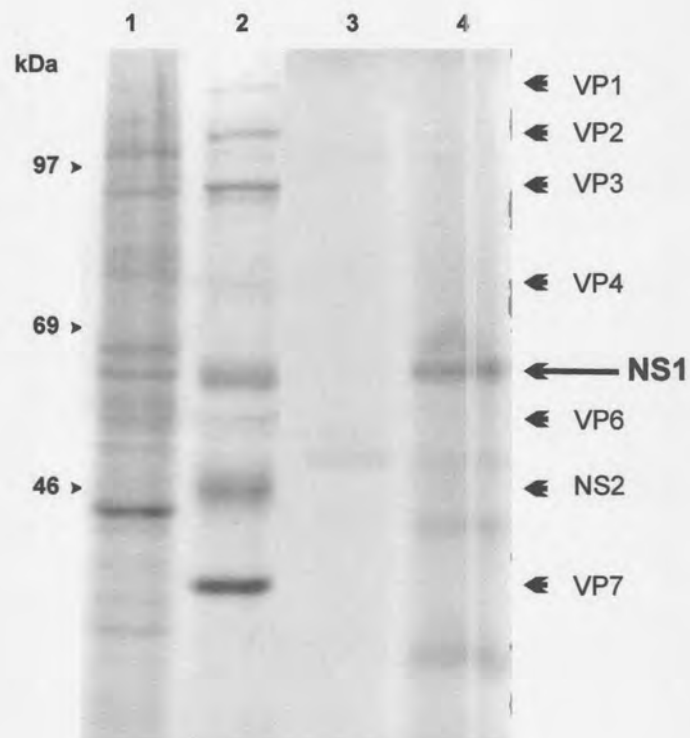


Figure 2.14: Autoradiograph of the ^{35}S -methionine labelled *in vitro* translation products directed by mRNA synthesised from AHSV-6 NS1 chimeric gene. Uninfected CER cells (lane 1) and CER cells infected with AHSV-3 (lane 2) were pulse labelled for 3 h with ^{35}S -methionine 16 h post-infection. *In vitro* translation of mRNA, synthesized from the NS1 gene directed the synthesis of a 63 kDa protein (lane 4) that corresponded in size to NS1 in AHSV-3-infected CER cells. Lane 3 represents rabbit reticulocyte translation products in the absence of any input mRNA.

2.3.5. Expression of the NS1 gene of AHSV-6 in insect cells by means of a recombinant baculovirus

2.3.5.1. Construction of a recombinant baculovirus

The BAC-TO-BAC™ baculovirus expression system, developed by Luckow *et al.* (1993), was used to express NS1 in insect cells. A recombinant baculovirus, containing the NS1 chimeric gene under control of the polyhedrin promoter, was constructed as outlined in *Figure 2.2C*. The NS1-specific insert was recovered from pBS-S5Hyb and cloned into the *Bam*H1 site, downstream of the polyhedrin promoter of the transfer vector, pFastbacI. A recombinant plasmid (pFB-S5Hyb), containing the NS1 gene in the correct transcriptional orientation, was identified by restriction mapping with *Hind*III and *Xho*I/*Xba*I. A plasmid yielding two *Hind*III fragments of approximately 1.2 kb and 5.4 kb and two *Xho*I/*Xba*I fragments of approximately 350 bp and 6.3 kb, as predicted for the NS1 gene to be in the correct orientation, was selected (*Figure 2.15*). A recombinant baculovirus shuttle vector (bacmid DNA) was then constructed by site-specific transposition of the cloned foreign gene into the baculoviral genome with the aid of the helper plasmid, which provides the transposition functions in *trans*. High molecular weight composite bacmid DNA was screened by dot-blot hybridisation and PCR amplification (*Figure 2.16*). Composite bacmid DNA, which yielded a 1.65 kb fragment with PCR and hybridised with segment 5-specific probe was transfected into Sf9 cells using a cationic lipid reagent (section 2.2.11.2). The supernatant containing the recombinant baculoviruses (Bac-AH6NS1) was harvested from transfected cells after 3-4 days. Progeny viruses were obtained by plaque-assay and used for the propagation of recombinant baculovirus stocks.

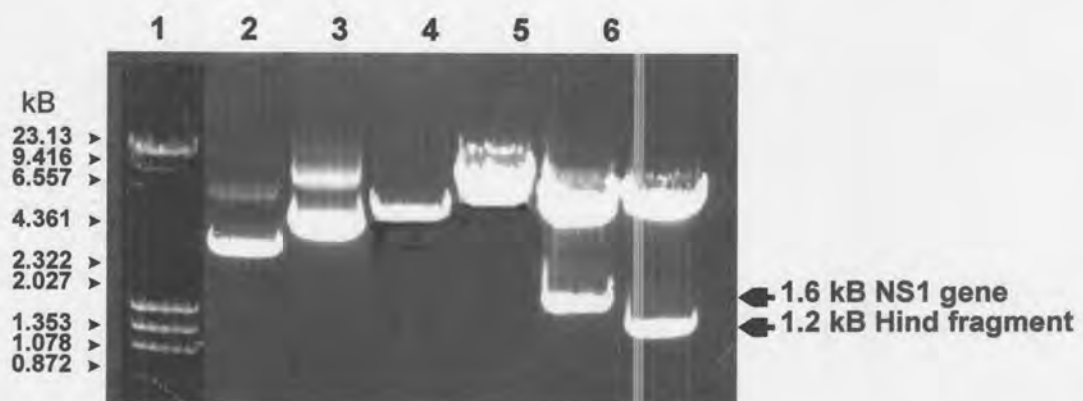


Figure 2.15: Agarose gel electrophoretic analysis of the recombinant plasmid pFB-S5.2Hybr, constructed by cloning the PCR/cDNA chimeric NS1 gene into the *Bam* H1 site of pFastbacI. Uncut pFB-S5.2Hybr (lane 3) was compared to uncut pFastbacI (lane 2) and was also restricted with *Xho* I (lane 5), *Bam* H1 (lane 6) and *Hind* III (lane 7). Linearised pFastbacI (lane 4) was included as a control. See figure 2.2 for R.E. map of NS1 gene. The sizes of MWII and ϕ X174 DNA are indicated to the left (lane 1) of the figure.

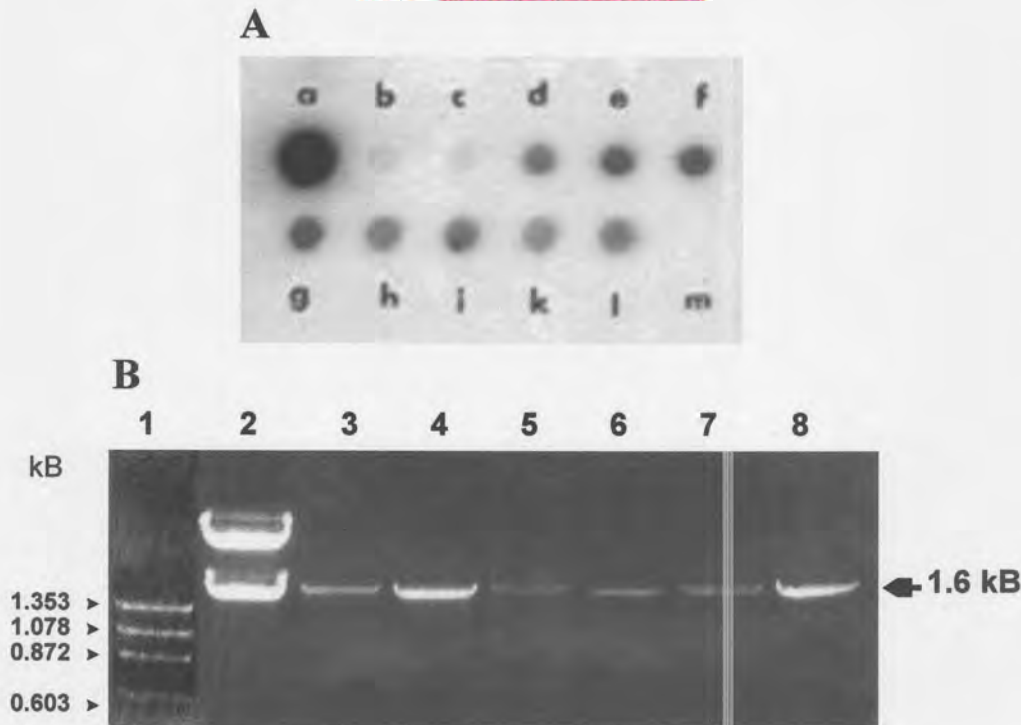


Figure 2.16: (A) An autoradiograph representing dot-blot hybridisation of a ^{32}P -labelled NS1-specific probe (pBR-S5.2) to recombinant bacmid DNA. The probe hybridised specifically to the NS1 gene. A positive control was pFB-S5.2Hybr (a) and DNA from blue bacmid colonies (b and c), and wild type pFastbac (m) served as negative controls. Dots d - l represent recombinant bacmid DNA which were selected for PCR amplification. (B) Agarose gel analysis of PCR amplified fragments from composite bacmids d - i (lanes 3 - 8). Lane 1 represent Hae III-digested ϕX174 DNA and lane 2 pFB-S5.2Hybr digested with Bam H1.

2.3.5.2. Expression and purification of AHSV-6 NS1 protein

Protein extracts were prepared from insect cells infected with the recombinant baculovirus, Bac-AH6NS1 and analysed by SDS-PAGE (Figure 2.17). The results revealed that the recombinant virus synthesised large amounts of a unique protein with a molecular size of 63 kDa, which is in agreement with the estimated size of the AHSV-6 NS1 protein. This protein was absent in mock- or wild type baculovirus-infected cells. To further confirm the identity of the 63 kDa protein, proteins synthesised in Bac-AH6NS1 infected Sf9 cells were pulse labelled with [^{35}S]methionine between 36 and 40 h p.i. and the labelled proteins were analysed by SDS-PAGE. Radio-labelled proteins from AHSV-3-infected CER cells were included for comparison. The unique protein expressed by BV-AHSV6-S5 was exactly the same size as the NS1 protein of AHSV-3 (Figure 2.18A). The yield of expressed NS1 in Bac-AH6NS1-infected cells was estimated to be approximately $1 \text{ mg}/10^6$ cells. It was not possible to confirm the viral origin of the putative NS1 by immuno-precipitation using rabbit anti-AHSV-6 antisera. The results were inconclusive since the NS1 protein was in a particulate form and precipitated under the same conditions as NS1-antibody complexes (results not shown). Electron microscopy analysis was used for confirmation (section 2.3.6).

The NS1 protein in lysates of cells infected with the recombinant baculovirus was subjected to sucrose gradient sedimentation analysis according to the method described by Huismans and Els (1979). Samples of each fraction from the discontinuous gradient were analysed by SDS-PAGE (Figure 2.18B). The results indicated that the largest amount of the 63 kDa protein was recovered from those sucrose gradient fractions in the region of 200-400 S, which suggests that the expressed protein is present in a particulate or polymerised form in the infected cells. A similar heterogeneous 400S complex of NS1 protein has been identified in AHSV-infected mammalian cells (Huismans & Els, 1979). The heterogeneity of the S values on sucrose gradients could be due to breakage during the purification process.

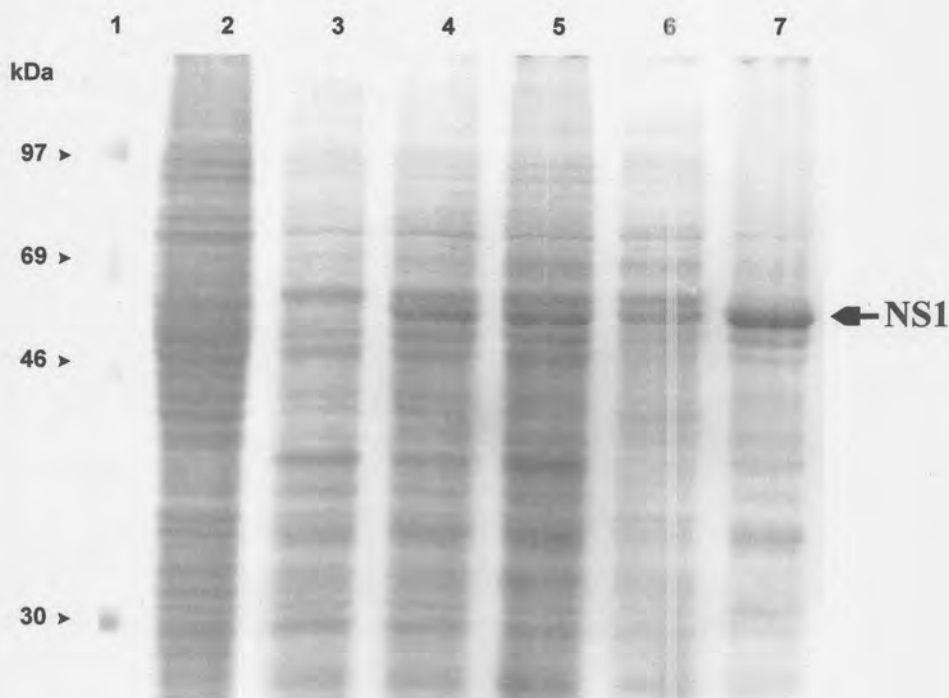


Figure 2.17: SDS-PAGE analysis of the expression of the NS1 protein in insect cells infected with a recombinant baculovirus containing the NS1 gene. *S. frugiperda* cells were mock-infected (lane 2), or infected with either the wild-type baculovirus (lane 3) or the recombinant baculovirus, Bac-AH6NS1 clones 3, 4 and 7 (lanes 4-6). Cells were harvested at 72 h post-infection and disrupted. Lane 7 represent partially purified NS1, by means of differential low speed centrifugation of cytoplasmic extracts of infected cells. Proteins were visualised after staining with Coomassie blue. The position of NS1 and molecular weight markers are indicated.

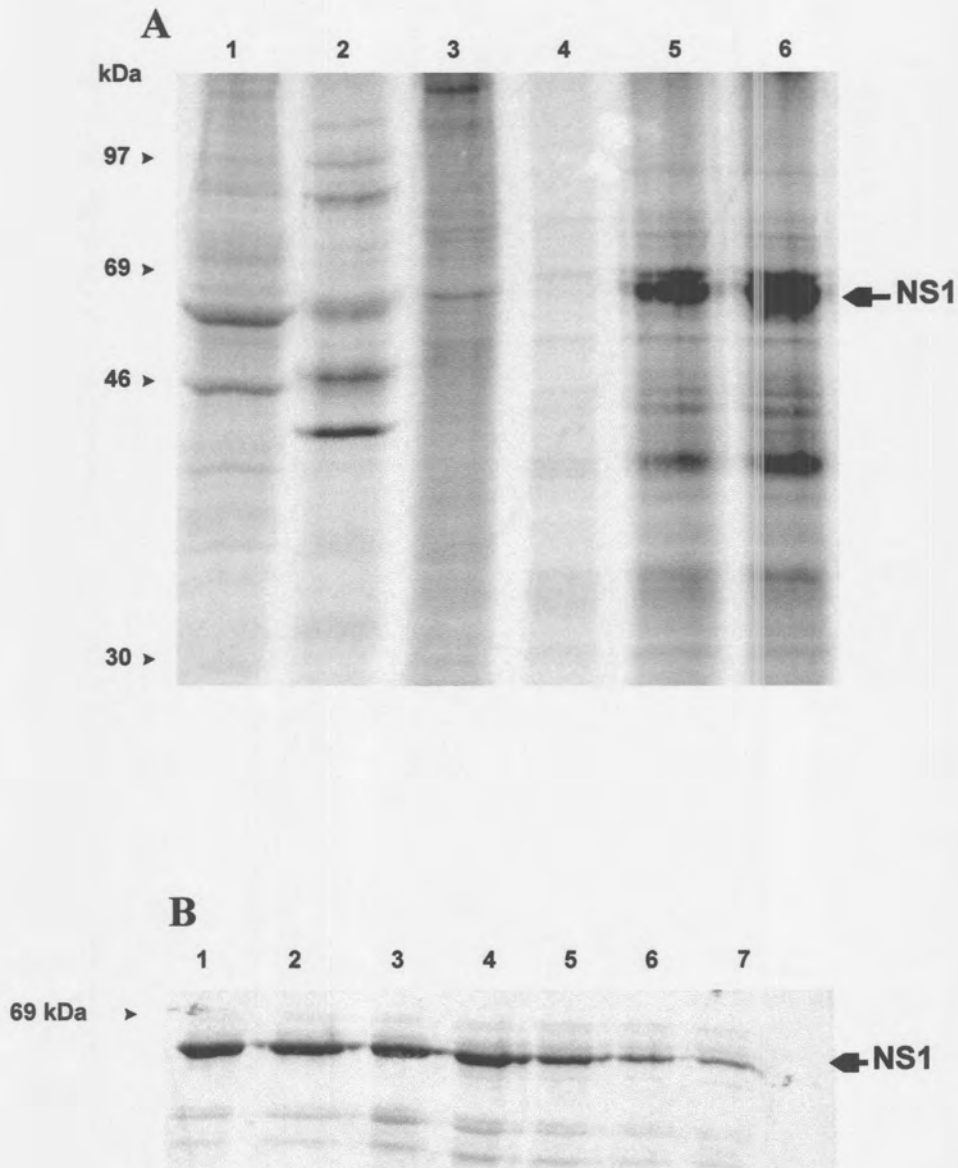


Figure 2.18: Autoradiograph of SDS-PAGE separated cell lysates of insect cells infected with recombinant and wild-type baculoviruses. Lanes 1 and 2 represent uninfected CER cells and CER cells infected with AHSV-3, pulse labelled for 3 h with ^{35}S -methionine, 16 h post-infection. Mock-infected (lane 3), wild-type infected (lane 4) and Bac-AH6NS1-infected (lanes 5 and 6) insect cells were pulse labelled 36 and 48 h post-infection. (B) Multimeric NS1 protein complexes were recovered by centrifugation through a 40% sucrose cushion and further purified by sedimentation on a 20-50% sucrose gradient. Fractions 4 to 10 (lanes 1-7) of 20 fractions, contained the NS1 protein complexes.

2.3.6. Electron microscopy of the NS1 protein complex

The 200-400 S NS1 complex was concentrated from sucrose gradient fractions by centrifugation for 2 h at 40 000 rpm. The pellet was resuspended in STE buffer and analysed by electron microscopy after negative staining. The results (*Figure 2.19A*) indicated that the material consisted almost exclusively of tubules of an average diameter of 23 ± 2 nm and various lengths of up to 4 μm . The variation in length of the NS1 tubules may reflect the normal variation in tubule length or could be due to breakage during the purification process. The fine structure of the AHSV tubules was very different in appearance to those of BTV and EHDV tubules. AHSV tubules had an internal structure with a fine reticular "cross-weave" appearance. The central region of the tubules was characterised by alternating stretches of electron dense and less dense areas. The edges of the tubules were smooth and sharply defined with no visible pattern of subunits. There was no evidence of the ladder-like or segmented appearance found in the much wider BTV (68 nm) and EHDV (52 nm) tubules. In addition to the tubules flat, hollow, circular structures were also observed. The diameter of these structures corresponded to that of the AHSV tubules while their lumen had a diameter of approximately 7 nm. These structures probably represent cross-sections through tubules.

A small percentage of baculovirus-specific tubules (result not shown) were also observed. These tubules could be distinguished from the AHSV-specified tubules by the fact that they have a diameter of 40 nm and also by differences in fine structure. The identity of the AHSV tubules was verified by means of antibody decorating, using AHSV-6 antiserum (described in section 2.2.15). This involves binding of AHSV-6 antiserum to tubules, immobilised on grids. Different dilutions of the antiserum were used and decorating was clearly observed with a dilution of up to 1/2000. The NS1 tubules (*Figure 2.19B & C*) were distinguished from the wild-type baculovirus tubules in that they appeared darkly shadowed as a result of the antiserum decoration.

The sucrose gradient purified tubules were also investigated at high resolution with In-lens FESEM to gain more insight on the surface structure of the tubules. The tubules were present as a network and could be distinguished from baculovirus tubules according to their diameter. These tubules have a smooth surface structure with no indentations and projections (*Figure 19D & E*). This is in agreement with the absence of a ladder-like appearance in transmission electron micrographs.

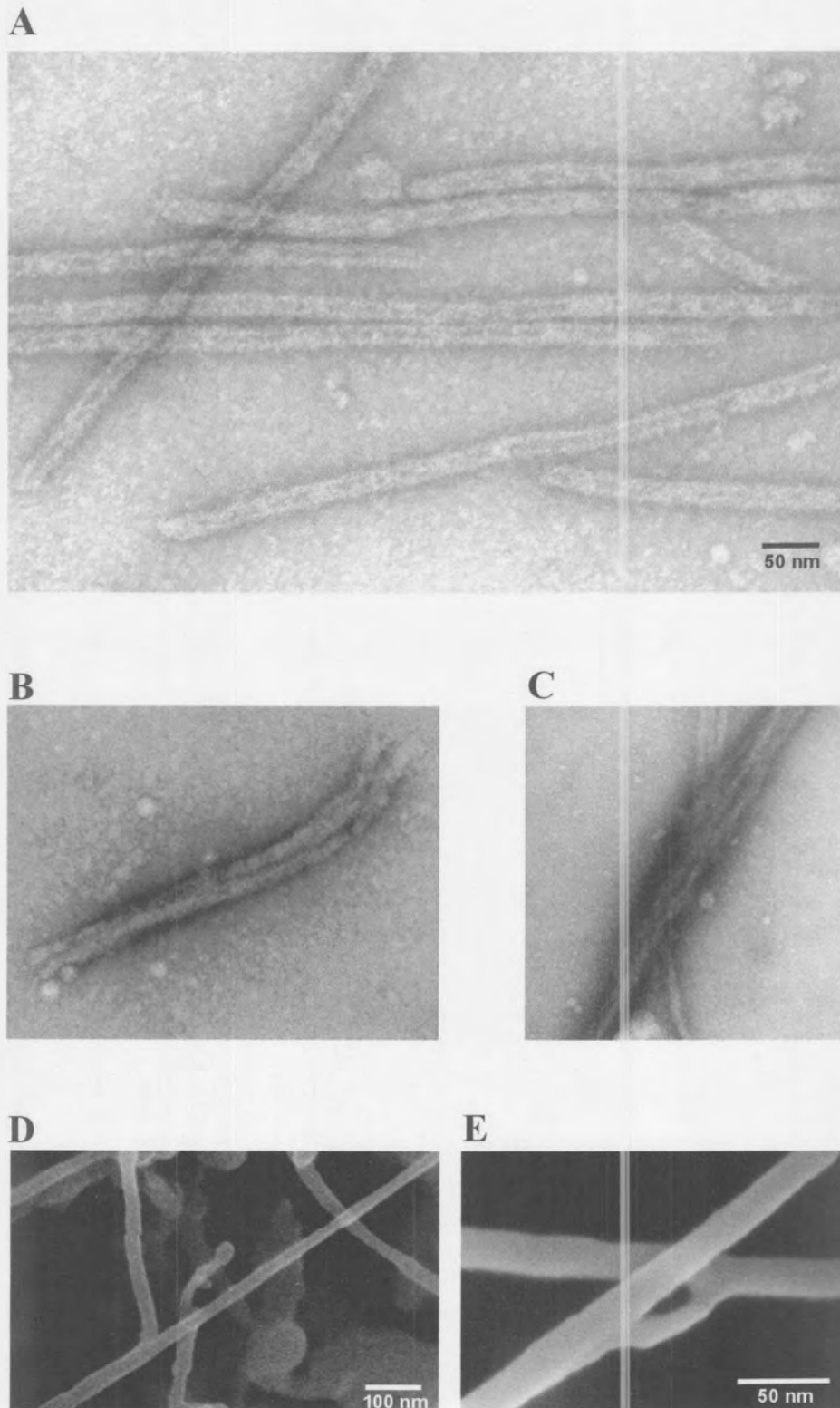


Figure 2.19: Negative contrast electron micrographs. The recombinant baculovirus-expressed NS1 tubules were purified by sucrose gradient centrifugation and stained with 2% uranyl acetate (A). The identity of the purified NS1 tubules were verified by decoration with anti-AHSV-6 antisera at 1:1000 (B) or 1:500 (C) dilutions before staining with uranyl acetate. The NS1 tubules could be distinguished from baculoviral tubule contaminants (not shown). Bar markers represents 50 nm. (D & E) In-lense SE micrographs of the surface of NS1 tubules. Bar markers represent 50 nm.

2.3.7. Electron microscopy of thin sections of recombinant baculovirus-infected cells

Recombinant baculovirus-infected Sf9 cells were fixed by glutaraldehyde treatment and thin sections prepared and processed for electron microscopy. The results are shown in *Figure 2.20*. Large amounts of tubules of various lengths and in different three-dimensional arrangements were observed in the recombinant virus-infected cells. These structures were absent in mock-infected (*Figure 2.20A*) and wild-type infected control cells. In recombinant virus-infected cells the tubules occurred mostly in the cytoplasm, but they have also occasionally been observed in the nucleus. Whether the tubules occurred in the nuclei because the nuclear membrane had been disrupted in the terminal stage of infection, is not known. In a few cases the tubules seemed to be associated with and aligned along unidentified fibrous material (*Figure 2.20B*). In most of the cell thin sections, the NS1 complexes could be seen as twisted and layered stacks and bundles, angular to each other with various configurations (*Figure 2.20C*). Both the structure and arrangement of the tubules were comparable to those of the tubular structures reported in AHSV-infected BHK cells (Huisman & Els, 1979). The number of tubules in the cytoplasm generally appeared to correlate directly with the number of viruses and virus-inclusion bodies in the enlarged nucleus of the recombinant virus-infected cells.

2.3.8. The effect of biophysical conditions on the morphology of AHSV NS1 tubules

The effects of different biophysical conditions on the morphology of the tubules were investigated and are summarised in *Table 2.5*. Aliquots of gradient purified tubules were resuspended in various test solutions and both the particulate and supernatant fractions analysed for the presence of NS1 protein. The fractions were also investigated by TEM. Tubule morphology was found to be affected by the ionic strength of the buffer solution in which it was resuspended. The tubules were found to be unstable at a CaCl₂ concentration of 0.2 M and higher, since NS1 was present in the pellet following low speed centrifugation. No tubules were observed under these conditions and only an amorphous mass of proteins was visible. In the presence of 1 M NaCl, most of the NS1 was retained in the supernatant. Although tubules could be observed, they appeared to be much shorter than normal (*Figure 2.21A*) and a large increase in the number of circular forms were observed. When treated with buffers of between 6.5 and 7.5, the tubule morphology was unaffected.

Treatment with buffers of between pH 8.0 and 8.5, resulted in severely affected tubule morphology (*Figure 2.21B*). The tubules were not only reduced in length but the fine structure was totally abolished and the NS1 protein became aggregated. Between pH 5.0 and 5.5 the tubules were generally of shorter length and the surface of the tubules appeared more uneven (*Figure 2.21C*). At pH 5.0 or less, the tubules appeared to be denatured and NS1 aggregated in an amorphous protein mass. At temperatures above 50°C, the NS1 was found in the pellet and the tubules were dissociated.

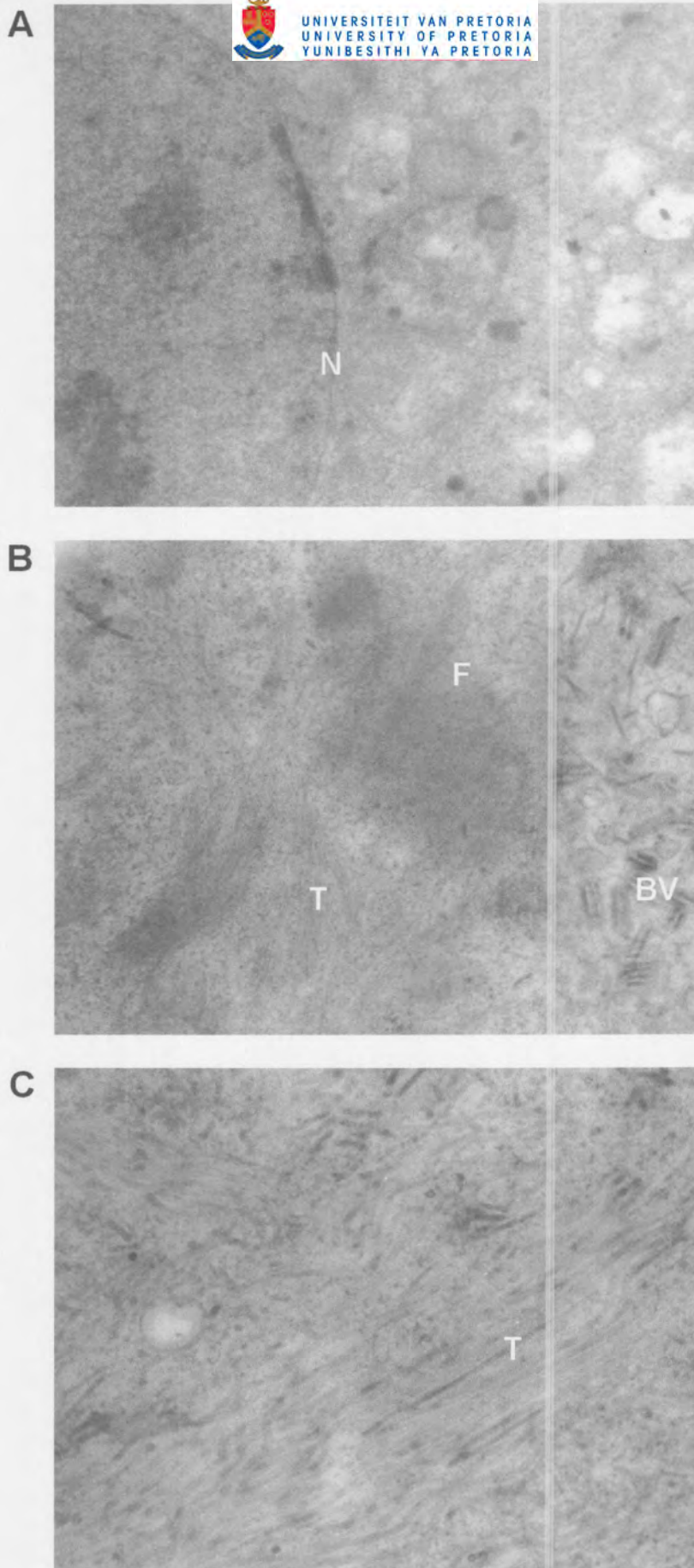


Figure 2.20: Negative contrast electron micrographs of thin sections of insect cells infected with the recombinant baculovirus Bac-AH6NS1. (A) represents an uninfected cells while (B) and (C) show bundles of NS1 tubules in the cytoplasm of infected cells. T, tubules; BV, baculovirus particles; F, fibrous material; N, nuclear membrane.

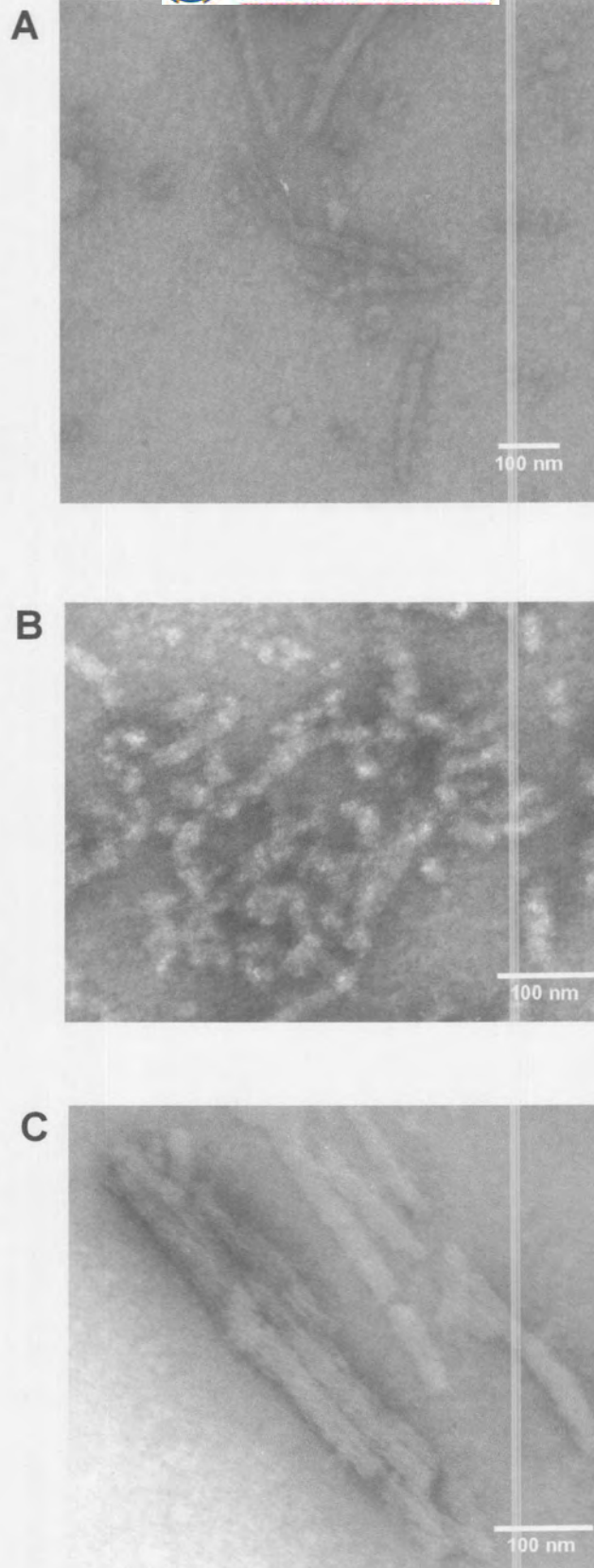


Figure 2.21: Electron micrographs of negatively stained tubules , treated with 1 M NaCl (A), buffer of pH > 8.0 (B) and buffer with pH 5.0 - 5.5 (C). Bar markers represents 100 nm.

TABLE 2.5: *Effects of different treatments on AHSV tubule morphology*

Treatment	Effect on morphology
pH	
<5.0	Tubules denatured; aggregated protein
6.0-8.0	Shortened tubules; filamentous strands
>8.0	Tubules denatured; aggregated fragments
Ionic strength	
0.2 M CaCl ₂ /MgCl ₂	Tubules denatured; aggregated protein
<1 M NaCl/KCl	Shortened tubules; circular formation
>1 M NaCl/KCl	Tubules denatured; aggregated protein
Detergents	
Anionic	Tubules denatured; filamentous strands
Cationic	Tubules denatured; aggregated protein
Non-ionic (Tween 20/Triton X-100)	Tubules denatured; aggregated protein
Chelating agents	
1-3 mM EDTA	Fragmented short tubules
4-5 mM EDTA	Circular formation; no tubules visible
Reducing agents	
100 mM DTT	Shortened tubules
5% (v/v) Mercaptoethanol	Shortened tubules
Denaturing conditions	
M Urea	Tubules denatured; aggregated protein
SDS treatment	Tubules denatured; aggregated protein
Temperature	
4°C	Normal tubules
30-45°C	Normal tubules
45-60°C	Shorter tubules with end filaments
60-70°C	Filamentous network

2.4. DISCUSSION

The first part of this investigation was focused on the cloning and characterisation, through nucleotide sequencing, of the dsRNA segment that encodes the non-structural protein NS1 of AHSV-6. This information not only provides insight into the properties of the gene and deduced NS1 protein but more important assists in identifying regions for the insertion of foreign epitopes and the understanding of tertiary structure of the protein. The AHSV-6 NS1 protein was also expressed *in vitro* and in a baculovirus-based eukaryotic expression system.

The relationship among three different AHSV serotypes was investigated by comparing the determined AHSV-6 segment 5 nucleotide sequence and deduced amino acid sequences to that of the cognate genes of AHSV-4 (Mizukoshi *et al.*, 1992) and 9 (Nel, unpublished data). The NS1 gene of AHSV-6 was found to be 1748 nucleotides in length and differed only in three nucleotides in the 3' non-coding region from the NS1 gene of serotype 4. The longest open reading frame observed, contained 548 codons flanked by 5' and 3' non-coding regions of 35

and 70 nucleotides respectively. The presumed translation initiation codon conformed fully to the eukaryotic consensus sequence (CX^A₆XCAUGG) proposed by Kozak (1987). The 5' and 3' termini of AHSV-6 NS1 were found to contain the characteristic conserved GTT^A₇AA and AC^A₇TAC terminal hexanucleotide sequences of AHSV RNA fragments proposed by Van Staden *et al.* (1991). These terminal sequences are a characteristic feature not only to AHSV, but to all orbivirus RNA segments investigated thus far (Rao *et al.*, 1983; Mertens & Sanger, 1985; Wilson *et al.*, 1990) although the sequence and which bases are conserved can vary between different *Orbivirus* species. A near identical inverted repeat sequence of 11 bp with a homology of more than 87% was identified in the 5' and 3' non-coding regions of AHSV NS1 gene. Short inverted repeat sequences adjacent to the 5' and 3' termini have been identified for all orbivirus dsRNA segments (Roy, 1989; Nel *et al.*, 1990; Anzola *et al.*, 1987; Steockle *et al.*, 1987; Moss *et al.*, 1992 a, b). It has been reported that the NS1 gene of EHDV contain a second inverted repeat sequence of 19 nucleotides in the 3' non-coding region in addition to the above described inverted repeats (Nel *et al.*, 1990). However, similar to the BTV NS1 gene no inverted repeat sequence was present in the 3' non-coding region of the NS1 gene of either AHSV-6 or 9. A comparison of the three cognate AHSV NS1 genes revealed a close relationship between AHSV-4 and 6 (97%) and somewhat less conservation between AHSV-6 and 9 (93%). The 5' non-coding region was found to be highly conserved among the three different serotypes, while less homology was observed in the 3' non-coding region. A comparison with the cognate NS1 gene (segment 5; 1769 bp long) of BTV-10/13/17 (Lee & Roy, 1987; Wang *et al.*, 1988) revealed 44% nucleotide similarity, which is in agreement with the results reported for AHSV-4 (Mizukoshi *et al.*, 1992). The major difference in the length of the AHSV and BTV NS1 genes is due to the additional 7 nucleotides in the non-coding 3' region of the BTV NS1 gene, whereas the length of the coding regions differs by only 12 nucleotides (or 4 codons). The 3' non-coding region of EHDV-2 (Nel *et al.*, 1990) is 46 nucleotides longer than that of AHSV-6 and 9. The 3' terminal of the NS1 genes of all three viruses, although of different lengths, share the feature of being rich in thymidine residues. The nucleotide homology of the coding regions among AHSV, BTV and EHDV was found to be 47%, 46% and 62% respectively. The 5' non-coding region is even more highly conserved. Conserved nucleotide sequence at the 5' non-coding region appear to be a general feature of cognate orbivirus genome segments (Nel *et al.*, 1990).

According to Nel *et al.* (1990), the high level of conservation of the 5' leader sequences could be a reflection of the importance of these regions in controlling the relative rate at which the genome segments are transcribed or the mRNA species are translated. It has been shown in the case of reovirus that the relative rate of mRNA translation is determined by the 5' leader sequence. A characteristic feature of the NS1 encoding genome segments of orbiviruses is the

observation that these genome segments are much more frequently transcribed than the other segments (Huisman & Verwoerd, 1973; Huisman *et al.*, 1979).

The NS1 gene of AHSV-6 specifies a protein of 548 amino acids in length with an estimated molecular mass of 63 478 Da and a net charge of +5 at neutral pH. This is in agreement with the NS1 protein of AHSV-4 ($M_r = 63\ 122$; Mizukoshi *et al.*, 1992) and 9 ($M_r = 63\ 378$), but is four amino acids shorter than the BTV and EHDV equivalent. AHSV-6, BTV-10 (13 and 17) and EHDV-2 proteins all comprise a similar number of amino acids. The AHSV-6 gene product is 548 amino acids in length, with BTV and EHDV-2 both consisting of 552 amino acids. The proteins have similar sizes, with M_r of 63 478, 64 445 and 64 559 for AHSV-6, BTV and EHDV, respectively. Like the NS1 protein of BTV (Lee and Roy, 1987; Wang *et al.*, 1989), AHSV-6 NS1 has a low content of charged amino acids, as well as threonine and serine residues, but is particularly rich in cysteine residues. Monastyskaya *et al.* (1994) indicated that at least some of the conserved cysteines are essential for tubule formation. Point mutations of cysteine residues 337 and/or 340 resulted in the failure to form tubules. These cysteines probably play a role in the formation of the correct 3-D conformation of NS1 because it was indicated that inter-chain disulphide bonds are not involved in maintaining the tubule structure (Marshall *et al.*, 1990). These two cysteines are located in a hydrophilic region of the protein, unlike many other conserved cysteine residues (Monastyskaya *et al.*, 1994).

A number of highly conserved regions (10-20 amino acids), containing approximately 70% identical amino acids, were identified in a comparison of the NS1 proteins of AHSV, BTV and EHDV (Figure 2). These conserved regions can further be reduced to a number of highly conserved amino acids which account for 9% of the total number of amino acids in a comparison of NS1 of AHSV, BTV, EHDV and BRDV (Moss & Nuttal, 1995). The AHSV-6 NS1 protein is, similar to AHSV-4 (Mizukoshi *et al.*, 1992), BTV (Lee and Roy, 1987) and EHDV NS1 (Nel *et al.*, 1990), predominantly hydrophobic with the hydrophobic residues accounting for as much as 43.6% of the total amino acids in NS1. This is significantly more than the 36-37% hydrophobic residues found in BTV and EHDV NS1 proteins. The hydrophobic profile of AHSV-6 NS1 protein shows some similarity with that of BTV and EHDV NS1, and revealed that with some exceptions in the hydrophilic regions, the NS1 protein from the three different viruses had almost identical distributions of hydrophobic and hydrophilic domains. The hydrophobic domains of AHSV, BTV and EHDV are located in areas of highest amino acid similarity and their location is almost exactly conserved in the NS1 proteins of the three orbiviruses. These hydrophobic stretches are also conserved in BRDV NS1 (Moss & Nuttal, 1994) and may play an important role in tubule formation. It is interesting to note that although the location of these

hydrophobic domains is highly conserved, the diameter of the tubules into which they respectively polymerise can differ significantly.

Monastyrskaya *et al.* (1995) reported that deletion mutants of BTV-10 NS1 of up to 10 amino acids in the amino terminus or 20 to 43 amino acids from the carboxy terminus, failed to form tubules. This indicates that regions of both the N- and C-termini are important for tubule formation. Removal of five C-terminal amino acids or addition of 16 amino acids did not impair the ability of the derived NS1 to form tubules. This is in agreement with the finding that the N- and C-termini of the NS1 protein are the most conserved among AHSV, BTV and EHDV. Mikhailov *et al.* (1996) demonstrated that peptides of up to 109 amino acids, inserted at the C-terminus of BTV-10 NS1, allowed the formation of tubular structures. When insect cells were co-infected with three recombinant baculoviruses expressing chimeric NS1 proteins with different epitopes, they simultaneously assembled into the same tubule. These chimeric tubules, carrying foreign antigenic peptides, were highly immunogenic, even in the absence of adjuvants. Using the antigenic profile (Welling *et al.*, 1989) in combination with the hydrophilicity profile (Kyte & Doolittle, 1982) of the deduced amino acid sequences of the different AHSV NS1 proteins, a strong antigenic site was identified in the C-terminal 100 amino acids of the NS1 protein. This site corresponds to an antigenic epitope that was identified on BTV NS1 at position 474 to 502 (Du Plessis *et al.*, 1995).

As part of an investigation into studying the polymerisation of NS1 into tubules, the NS1 protein of AHSV-6 were expressed *in vitro* as well as in insect cells, using an improved baculovirus expression system. The level of NS1 expression *in vitro* was relative high, which is in agreement with the level at which NS1 is expressed in AHSV-infected cells (Huisman & Verwoerd, 1973; Venter, 1991). We have obtained evidence that the *in vitro* synthesised NS1 protein of AHSV forms small tubular structures, similar to those in recombinant baculovirus-infected cells. Although the structure of the *in vitro* synthesised tubules appears to be less homogenous than that of authentic or cell culture-produced AHSV NS1 tubules, it could be ascribed to pH or other physical conditions. This indicates that the ability of NS1 to form tubules of specific structure and diameter is primarily a function of the structural units as determined by the specific amino acid sequence. Nel *et al.* (1992) also reported the synthesis of tubules after *in vitro* translation of EHDV NS1. The NS1 protein of EHDV is predominantly present in a particulate form even when small amounts are synthesised, which indicates that the condensation of the NS1 into tubules occurs very rapidly (Nel & Huisman, 1991). This indicated that the ability to form tubules is self-primed and as such a function of the amino acid sequence of the NS1 protein.

To resolve the structure of AHSV-specified tubules, NS1 was expressed to a high level in insect cells by means of a recombinant baculovirus. The *in vivo* synthesised NS1 protein was present in a particulate or polymerised form that could be isolated on sucrose gradients as a tubular complex with a sedimentation value which was in agreement with that of authentic NS1 tubules from AHSV-infected CER cells (Huismans & Els, 1979). Electron microscopic analysis of the NS1 protein complexes indicated that they contained the characteristic AHSV tubules, with a similar morphology as described for authentic tubules isolated from AHSV-3 infected cells (Huismans & Els, 1979). This structure differed in many respects from those described for other orbiviruses such as BTV, EHDV (Huismans & Els, 1979; Urakawa & Roy, 1989; Nel & Huismans, 1991) and BRDV (Moss & Nuttal, 1994). The AHSV tubules were found to be 23 ± 2 nm in diameter as compared to the 68 nm diameter of BTV NS1 tubules (Marshall *et al.*, 1990). They varied in length, but tubules of up to 4 μ m were found. When the fine structure of these tubules was studied it became clear that there was no detectable ladder-like surface structure with linear periodicity as had been observed in the case of BTV, EHDV and BRDV (Urakawa & Roy, 1989; Nel & Huismans, 1991; Moss & Nuttal, 1995). No specific subunits were observed as in BTV and EHDV. Circular, flat, hollow structures were often seen which could represent cross-sections of tubules or very short subsections of larger tubules. Such circular forms have been reported to be an intermediate between BTV NS1 monomers and tubules and were shown to be able to recondense into tubules under appropriate conditions (Marshall *et al.*, 1990). The diameter of these circular structures was measured and corresponded to that of the tubules. The lumens of these circular forms were measured to be approximately 7 nm. Nel & Huismans (1991) described the difference in appearance of EHDV tubules stained with phosphotungstic acid (PTA) and uranyl acetate (UA). AHSV NS1 tubules were extremely unstable in the presence of PTA and no tubules were visible after staining with PTA, even at physiological pH and salt conditions. In-lens FESEM revealed a network of tubules. The surface structure of the NS1 tubules forms a continuity and no segments are visible.

Electron micrographs of thin sections of cells infected with the baculovirus NS1 recombinant indicated that the tubules occurred in the cytoplasm as bundles with random orientation. Urakawa & Roy (1988) have reported a close association of bundles of fibrous material with the tubules that are formed in *S. frugiperda* cells infected with a recombinant baculovirus that express the NS1 gene of BTV-10. In the case of BTV-infected cells, it has been shown that some but not all of the BTV tubules, together with a proportion of virus inclusion bodies and virus-like particles are cytoskeleton-associated (Eaton *et al.*, 1987; Eaton *et al.*, 1988; Hyatt & Eaton, 1988; Eaton *et al.*, 1990). It has also been suggested that NS1-rich fibrillar material condenses to form tubules after NS1 has been utilised in the process of virus morphogenesis (Eaton *et al.*, 1988). If soluble NS1 plays a role in viral morphogenesis, the condensation of

NS1 into tubules may only occur after a critical concentration of soluble NS1 has been reached. On the other hand it is possible that NS1 monomers are very rapidly added to the growing tubule filaments, after synthesis. In order to investigate the polymerisation of NS1 into virus-specific tubules, Nel & Huismans (1991) compared the ratio of particulate and soluble NS1 at different levels of expression. It was found that, even when a very small amount of NS1 was synthesised, almost all the NS1 was present in the particulate form.

Three dimensional studies of BTV NS1 tubules indicated that each tubule is composed of a coiled ribbon of NS1 dimers with 22 dimers per helix turn (Hewat *et al.*, 1992), while EHDV tubules contains only approximately 16 subunits per turn (Nel & Huismans, 1991). Although no clearly defined subunits in the AHSV tubules were identified, it could be illustrated that the AHSV NS1 tubules were more easily disrupted at a high ionic strength and at different pH conditions than BTV tubules. Marshall *et al.* (1990) reported that BTV tubules are reduced in length in a 0.5 M CaCl₂ solution while the AHSV tubules were completely denatured at 0.2 M CaCl₂. AHSV tubules were furthermore significantly reduced in length in 1 M NaCl. As in the case of BTV tubules (Marshall *et al.*, 1990), AHSV tubules were relatively resistant to low pH, but at an alkaline pH of 8.5 they were more susceptible to degradation than BTV tubules. The biophysical properties of AHSV NS1 versus BTV NS1 are summarised in *Table 2.6*. These results would suggest that AHSV tubules are more fragile than BTV tubules.

TABLE 2.6: *Biophysical properties of AHSV NS1 versus BTV NS1*

Property	AHSV tubules	BTV tubules
Acid sensitivity	Relative stable	Even more stable
Alkaline sensitivity	Extremely sensitive	Relative stable
Low ionic strength	Extremely sensitive	Relative stable
High ionic strength	Stable	Stable
Low temperature	Stable	Stable
High temperature	Sensitive	Sensitive
Ionic detergents	Extremely sensitive	Sensitive
Non-ionic detergent	Extremely sensitive	Stable
Chelating agents	Sensitive	Sensitive

There is as yet no evidence for a specific function of NS1 tubules in virus replication, although they may play a role in transport of mature virus particles from the virus inclusion bodies to the cell membrane where NS3 is involved in virus release. Although the amino acid sequence of NS1 and the structure and biophysical character of the tubules differ significantly among AHSV, BTV, EHDV and BRDV, their function may be conserved. Their synthesis is, however, a

characteristic feature of all orbiviruses investigated thus far. Marshall *et al.* (1990) has reported some similarities in the quaternary structure of BTV tubules and tubulin. Many different viruses such as rotaviruses (Kimura & Murakami, 1977), SV40 (Murphy *et al.*, 1986), reovirus (Dales *et al.*, 1963), Sendai virus (Moyer *et al.*, 1986) and others have been reported to be associated with or dependent on microtubules or microtubule-associated proteins (Hill & Summers, 1990) at some stage in their life cycle. Some evidence has accumulated that the formation of orbivirus tubules is an important event, which starts early in the infection cycle. In BTV-infected cells the NS1 mRNA is synthesised in a large excess in the very early period of the infection cycle (Huismans & Verwoerd, 1973; Huismans *et al.*, 1979). It has also been shown that tubules appear as early as 2 to 4 hours p.i. (Huismans & Els, 1979), are present in large numbers at 6 h p.i. (Eaton *et al.*, 1990) and that NS1 is synthesised in a significant excess when compared to the other viral proteins (Huismans, 1979). These results all indicate that NS1 is required in large amounts soon after infection.

Two unique antigenic regions of BTV NS1 were previously identified, one of which is located in the C-terminal hydrophilic region stretching from amino acids 470 to 500 (Du Plessis *et al.*, 1995). This region seems suitable for the insertion of epitopes in the construction of chimeric AHSV NS1 tubules. It is not known if the insertion of a foreign epitope in AHSV NS1 would influence the ability of AHSV NS1 to form tubular structures. It was shown in the literature that chimeric BTV NS1 tubules are highly immunogenic (Mikhailov *et al.*, 1996). NS1 tubules have many advantages over CLPs as epitope carriers. They are composed of a single protein, in contrast to CLPs, which consist of VP3 and VP7. Further, the expression level of the recombinant NS1 is generally extremely high (ca. 25% of the proteins synthesised in infected Sf cells)(Urakawa & Roy, 1988). Tubules are also easy to purify, and so they could provide an efficient and inexpensive system for the presentation of single or multiple foreign epitopes. It is furthermore likely that the use of multiple baculovirus gene expression vectors would increase the efficacy of formation of multiple epitope tubules, although this is yet to be investigated.
