CLONING, CHARACTERIZATION AND EXPRESSION OF THE GENE THAT ENCODES THE MAJOR NEUTRALIZATION-SPECIFIC ANTIGEN OF AFRICAN HORSESICKNESS VIRUS SEROTYPE 3.

by

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Even youths grow tired and weary,
and young men stumble and fall;
but those who hope in the Lord will renew their strength.
They will soar on wings like eagles;
they will run and not grow weary,
they will walk and not be faint.
(Isaiah 40 vv 30-31)

dedicated to my parents

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SUMMARY

Cloning, characterization and expression of the gene that encodes the major neutralization-specific antigen of African horsesickness virus serotype 3.

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The aim of this investigation was to clone, characterize and express the gene that encodes the outer capsid protein, VP2, of African horsesickness virus (AHSV), with a view to the evaluation of this protein as a subunit vaccine.

The VP2 gene of AHSV serotype 3 (AHSV-3) was cloned as incomplete cDNA fragments of the genome segment 2 double-stranded (ds)RNA, sequenced in its entirety and compared with previously published cognate sequences of AHSV-4, Epizootic hemorrhagic disease virus (EHDV)-1 and various bluetongue virus (BTV) serotypes. AHSV-3 genome segment 2 was shown to be 3221 nucleotides in length, encoding a protein of 1057 amino acids with a 50.5% identity to AHSV-4 VP2. Two areas of high variability (approximately 65%) were identified adjacent to the conserved termini. The N-proximal region (amino acids 128-309) exhibited significant hydrophilicity, suggesting a possible role in the determination of the serotype-specific immune response. Orbivirus interserogroup comparisons of VP2 amino acid sequences revealed extreme variability, although an overall structural conservation was demonstrated.

Oligonucleotide primers derived from the AHSV-3 genome segment 2 terminal nucleotide sequences were used for PCR amplification and cloning of full length segment 2 cDNA. The cloned gene was expressed in a baculovirus expression system and the expressed VP2 protein was shown to react specifically with anti AHSV-3 serum in Western blots.

Although the yields of VP2 in the baculovirus system were low, due to a possible toxic effect on the host cells, sufficient antigen was obtained for further future investigations into

the efficacy of VP2 as a possible subunit vaccine against AHSV.

OPSOMMING

Klonering, karakterisering en uitdrukking van die geen wat kodeer vir die hoof neutralisering-spesifieke antigeen van perdesiekte virus serotipe 3.

deur

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Die doel van hierdie ondersoek was om die geen wat vir die buite kapsied proteïen, VP2, van perdesiekte virus (PSV) kodeer, te kloneer, te karakteriseer en uit te druk, met die oog op die evaluasie van die proteïen as 'n subeenheid entstof.

Die VP2 geen van PSV serotipe 3 (PSV-3) is gekloneer as onvolledige cDNA fragmente van die genoom segment 2 dubbeldraad (dd)RNA, die nukleotiedvolgorde is in geheel bepaal en vergelyk met voorheen gepubliseerde ooreenstemmende volgordes van PSV-4, Epizootic hemorrhagic disease virus (EHDV)-1 en verskeie bloutong virus (BTV) serotipes. Die lengte van PSV-3 genoom segment 2 is bepaal op 3221 basispare (bp), wat kodeer vir 'n proteïen van 1057 aminosure, met 'n 50.5% identiteit aan PSV-4 VP2. Twee gebiede met 'n hoë variabiliteit (ongeveer 65%) is geidentifiseer langs die gekonserveerde ente. Die N-proksimale gebied (aminosure 128 tot 309) het betekenisvolle hidrofilisiteit getoon, aanduidend van 'n moontlike rol in die bepaling van die serotipe-spesifieke immuunrespons. Orbivirus interserogroep vergelykings van die VP2 aminosuur volgordes het uiterste variabiliteit geopenbaar, alhoewel daar 'n algehele strukturele konservering aangedui is.

Oligonukleotied voorvoerders, afgelei van die PSV-3 genoom segment 2 terminale nukleotied volgordes, is gebruik vir polimerase ketting reaksie (PKR) amplifikasie en klonering van vollengte segment 2 cDNA. Die gekloneerde geen is uitgedruk in 'n baculovirus uitdrukkingssisteem en die uitgedrukte VP2 proteïen is getoon om spesifiek met anti PSV-3 serum te reageer in Westelike kladde.

Alhoewel die opbrengste van VP2 in die baculovirussisteem laag was, agv van 'n

moontlike toksiese effek op die gasheer selle, is daar genoegsame antigeen verkry vir verdere toekomstige ondersoeke na die geskiktheid van VP2 as 'n moontlike subeenheid entstof teen PSV.

ABBREVIATIONS

A	-	adenosine
AHS	-	African horsesickness
AHSV	-	African horsesickness virus
AHSV-3	-	African horsesickness virus serotype 3
amp	-	ampicillin
AMV	-	avian myeloblastosis virus
ATCC	-	American type culture collection
ATP	-	adenosine-5'-triphosphate
BHK-21	-	baby hamster kidney cells
BHV-1	-	bovine herpes virus
bp	-	base pairs
BSA	-	bovine serum albumin
BT	-	bluetongue
BTV	-	bluetongue virus
°C	-	degrees Celsius
cDNA	-	complementary DNA
CF	-	complement fixation
Ci	-	Curie
CIP	-	calf intestinal alkaline phosphatase
CLP	-	core-like particle
CTL	-	cytotoxic T lymphocyte
cm	-	centimetre
cpm	-	counts per minute
dA	-	deoxyadenosine
dC	-	deoxycytidine
dG	-	deoxyguanosine
dT	-	deoxythymidine
dATP	-	2'-deoxyadenosine-5'-triphosphate
dCTP	-	2'-deoxycytidine-5'-triphosphate
dGTP	-	2'-deoxyguanosine-5'-triphosphate
dTTP	-	2'-deoxythymidine-5'-triphosphate
dNTP	-	2'-deoxynucleoside-5'-triphosphate
ddATP	-	2',3'-dideoxyadenosine-5'-triphosphate
ddCTP	-	2',3'-dideoxycytidine-5'-triphosphate
ddGTP	-	2',3'-dideoxyguanosine-5'-triphosphate
ddTTP	-	2',3'-dideoxythymidine-5'-triphosphate
ddNTP	-	2',3'-dideoxynucleoside-5'-triphosphate
DNA	-	deoxyribonucleic acid
ds	-	double stranded
dd	-	dubbeldraad
DTT	-	1,4-dithiothreitol
EDTA	-	ethylenediaminetetra-acetic acid
EHDV	-	epizootic hemorrhagic disease virus

EHV	-	equine herpes virus
et al.	-	et alia (and others)
FCS	-	fetal calf serum
Fig.	-	figure
FMD	-	foot and mouth disease
FMDV	-	foot and mouth disease virus
g	-	gram / gravitational acceleration
h	-	hour
HSV	-	herpes simplex virus
IBRV	-	infectious bovine rhinotracheitis virus
IgG	-	immunoglobulin class G
IPTG	-	isopropyl-ß-D-thiogalactopyranoside
kb	-	kilobase pairs
kDa	-	kilodalton
LB	-	Luria-Bertani
log	-	logarithmic
M	-	Molar
MAb	-	monoclonal antibody
MAK	-	methylated albumin Kieselguhr
mCi	-	millicurie
mg	-	milligram
min	-	minute
ml	-	millilitre
mM	-	millimolar
mmol	-	millimole
MMOH	-	methylmercuric hydroxide
MOI	-	multiplicity of infection
m/v	-	mass per volume
NaAc	-	sodium acetate
nm	-	nanometre
NS	-	non-structural
occ^+	-	occlusion positive
occ^-	-	occlusion negative
OD_{550}	-	optical density at 550nm
ORF	-	open reading frame
OVI	-	Onderstepoort Veterinary Institute
PAGE	-	polyacrylamide gel electrophoresis
PCR	-	polymerase chain reaction
pfu	-	plaque forming units
pi	-	post infection
PKR	-	polimerase ketting reaksie
RE	-	restriction endonuclease
RF	-	replicative form
RNA	-	ribonucleic acid
rpm	-	revolutions per minute

RPV	-	rinderpest virus
RT	-	room temperature
s	-	second
SCID	-	severe combined immunodeficient
SDS	-	sodium dodecyl sulphate
Sf9	-	*Spodoptera frugiperda*
ss	-	single stranded
TdT	-	terminal deoxynucleotidyl transferase
tet	-	tetracycline hydrochloride
TEMED	-	N,N,N',N'-tetramethylethylenediamine
T_H	-	T helper lymphocytes
Tris	-	Tris(hydroxymethyl)-aminomethane
U	-	units
µCi	-	microcurie
µg	-	microgram
µl	-	microlitre
UV	-	ultraviolet
V	-	volts
VIB	-	viral inclusion body
VLP	-	virus-like particle
VP	-	viral protein
v/v	-	volume per volume
WTV	-	wound tumor virus
X-gal	-	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside

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CHAPTER 1

LITERATURE SURVEY

1.1 AHSV CLASSIFICATION AND EPIDEMIOLOGY

African horsesickness (AHS) is an often fatal, infectious but non-contagious, arthropod-borne, viral disease of *Equidae*. The aetiological agent, African horsesickness virus (AHSV), is classified as a member of the *Orbivirus* genus of the family *Reoviridae* (Verwoerd *et al.* 1979). Other genera in the family include *Orthoreovirus*, *Rotavirus*, *Phytoreovirus*, *Fijivirus*, *Cypovirus* and *Coltivirus* (Matthews 1982; Francki *et al.* 1991; Gorman 1992). These viruses possess a segmented double-stranded (ds) RNA genome (generally of 10-12 segments) enclosed within a double-layered capsid (Gorman 1992; Matthews 1982; Fraenkel-Conrat *et al.* 1988).

Like the rota- and reoviruses, the orbiviruses are nonenveloped viruses with a diameter of 65-70 nm. Morphologically, the orbiviruses can be distinguished 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 1969; Borden *et al.* 1971; Murphy *et al.* 1971). The genus name is derived from the Latin *orbis*, a ring or circle. Members of the genus are subdivided into serogroups based on the cross-reactivity of their antigens in complement fixation (CF), immunodiffusion and immunofluorescence tests (Knudson & Monath 1990; Brown *et al.* 1991). Serotypes within a serogroup are recognized by distinct reactivities in serum neutralization tests. Nine different serotypes of AHSV have been identified (McIntosh 1958; Howell 1962).

AHS is endemic to sub-Saharan Africa (Brown & Dardiri 1990; House *et al.* 1992a), although severe epizootics have occurred in North Africa, the Middle East and in certain European countries (Mellor 1993). The most recent outbreaks have been reported in Spain, Portugal, Morocco and Saudi Arabia (House *et al.* 1992a; Mellor 1993, and references therein).

The virus is transmitted principally by arthropods of the *Culicoides* genus, although the transmission of AHSV through artificially infected mosquitoes and ticks has been reported (Mellor 1993). However, their role in natural transmission is not known.

AHSV infects horses, ponies, mules, donkeys and zebras (Brown & Dardiri 1990). Mortality from AHSV infection in horses is high. 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 carries (Davies & Otieno 1977; Erasmus *et al.* 1978; Brown & Dardiri 1990). Dogs have been reported to be infected with the virus, but their importance to the epidemiology of AHS has not been defined (McIntosh 1955; Salama *et al.* 1981). Until recently, AHSV was not known to infect man. However, AHSV has recently been implicated as the aetiological agent in 4 cases of encephalitis and chorioretinitis which occurred among laboratory workers in the vaccine-packing facility at the Onderstepoort Veterinary Institute (OVI), Pretoria, South Africa over a period of 8 years (Reid *et al.* 1992, Van der Meyden *et al.* 1992). It was postulated that the workers, who exhibited significant plaque reduction antibody titres to some of the strains incorporated in AHS vaccine, particularly to serotypes 1 and 6, were exposed to aerosol infection with neurotropic AHSV strains as a result of accidental breakages of bottles of freezedried vaccine. This was the first description of subclinical and probable clinical neurotropic AHSV infection in man, and constituted the first evidence that humans are susceptible to infection with neuroadapted strains of the virus (Swanepoel *et al.* 1992). This was subsequently confirmed by the successful experimental infection of primates (vervet monkeys) with vaccine strains of AHSV types 1 and 6 (Taylor *et al.* 1992).

Classically, the disease presents in four clinopathological forms (syndromes) in horses, which vary in the organs affected, the severity of lesions, the time of onset of clinical signs and mortality rates (Brown & Dardiri 1990). The pulmonary form is an acute disease characterised by high fever, severe pulmonary oedema, pleural effusion and mortality approaching 100% of affected horses. Cardiac AHS is subacute and results in pronounced oedema of subcutaneous and intermuscular tissues of the head and neck, accumulation of large amounts of fluid in the pericardial sac and multivocal haemorrhages of the epi-, endo- and myocardium. The mortality rate is 50-80%, but some animals do recover. A syndrome with features of both the pulmonary and cardiac forms, the so-called mixed form, has also been described and represents the most common presentation. The fever form is a mild to subclinical disease that most often develops in partially immune or resistant animals. Affected horses develop transient mild fever, some scleral infection and mild depression, but all recover. Recent results of studies by Laegreid *et al.* (1993) clearly indicated that in naive horses, the clinical form of the disease is a property of the AHSV inoculum.

In view of the serious implications of AHS, the need for an effective producer- and user-friendly vaccine is clear. By way of introduction, the following survey of literature will attempt to briefly outline the present knowledge concerning the structural and functional relationships of the relevant orbiviral virion proteins and to review the advances made in vaccine development against orbiviruses in particular, as well as against other members of the *Reoviridae* and other virus families.

1.2 AHSV MOLECULAR BIOLOGY

Little information is available about the molecular biology of AHSV. However, electron microscopic evidence and physicochemical studies indicate a close morphological and biochemical relationship with bluetongue virus (BTV), the prototype orbivirus (Oellerman 1970; Bremer 1976), which has been thoroughly investigated. Indeed, much of the current knowledge and research on AHSV is based on findings from studies of BTV.

1.2.1 AHSV virion

Like BTV, the AHSV virion consists of a double-layered protein capsid containing 10 dsRNA genome segments each encoding at least one viral protein (Grubman & Lewis 1992). The outer capsid is composed of two major proteins (VP2 and VP5) surrounding an icosahedral core particle composed of two major proteins (VP3 and VP7) and a subcore of three minor proteins (VP1, VP4 and VP6). In addition, at least two major (NS1 and NS2) and variable amounts of two minor (NS3 and NS3a) non-structural viral proteins have been identified in infected cells (Grubman & Lewis 1992; O'Hara *et al.* 1993).

Recent advances in cryo-electron microscopy and image analysis, as well as the ability to synthesize BTV core-like particles (CLPs) and virus-like particles (VLPs) using baculovirus expression vectors, have greatly facilitated an understanding of the virion architecture. The inner shell of core particles is apparently composed of 60 copies of VP3 arranged as 12 closely bonded pentamers. Pores in the core particles are essentially spaces between VP3 molecules, which could allow the passage of metabolites and RNA to and from the core for RNA transcription during infection. Between the pores and encapsidating the subcore, 260 spikes, attributed to VP7 trimers, appear as triangular columns with distinct inner and outer domains, forming the attachment sites for the two surface proteins. The VP5 proteins exist as globular structures within the channels formed by the rings of the VP7 trimers, predominantly hidden by the surface VP2 trimers which form sail-like spikes projecting beyond VP5. Inside the subcore, VP3 acts as a framework for interaction with the minor proteins VP1, VP4 and VP6 (French & Roy 1990; French *et al.* 1990; Le Blois *et al.* 1991; Loudon & Roy 1991; Hewat *et al.* 1992; Liu *et al.* 1992; Prasad *et al.* 1992).

1.2.2 AHSV genome

While noting important differences from the coding assignments of AHSV-4 previously reported by Grubman & Lewis (1992), O'Hara *et al.* (1993) found by *in vitro* translation

analyses that the coding assignments of AHSV-4 are consistent with those reported for BTV, namely that RNA segments 1, 2, 3, 4, 5, 7 and 9 encode VP1, 2, 3, 4, 5, 7 and 6, segments 6 and 8 encode NS1 and 2 and segment 10 encodes NS3 and 3a respectively.

The recent availability of nucleotide sequences of genomic segments of a number of different orbiviruses have made detailed comparisons of genes and gene products possible, in addition to enabling 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 AHSV RNA segments 2, 3, 4, 5, 6, 7, 8 and 10 have been determined and their similarities with the analogous genes of BTV or other orbiviruses documented (Roy *et al.* 1991; Van Staden & Huismans 1991; Van Staden *et al.* 1991; Iwata *et al.* 1992; Mizukoshi *et al.* 1992).

1.2.3 AHSV antigenic structural proteins

The proteins that elicit the major antibody responses, both in vaccinated and naturally AHSV-infected horses, have been shown to be three structural proteins, VP2, VP5 and VP7 and the four non-structural proteins, NS1, NS2, NS3 and NS3a (Laviada *et al.* 1993). VP2, the major outer capsid protein, has been established as the most variable component of the orbiviruses characterized to date (Mertens *et al.* 1984; Le Blois *et al.* 1991; Iwata *et al.* 1992) and has been identified as the primary viral protein responsible for inducing serotype-specific, neutralizing antibodies capable of conferring protective immunity in BTV (Huismans & Erasmus 1981; Kahlon *et al.* 1983; Huismans *et al.* 1987b; Inumaru & Roy 1987; Gould *et al.* 1988; White & Eaton 1990) and Palyam virus (Whistler & Swanepoel 1988).

Although amino acid sequence comparisons, secondary structure predictions and hydropathy analyses of VP2 of different BTV serotypes have been inconclusive in identifying potential antigenic sites (Fukusho *et al.* 1987; Ghiasi *et al.* 1987; Gould 1988; Yamaguchi *et al.* 1988), evidence for the existence and location of several neutralization-specific epitopes on VP2 of various BTV serotypes has accumulated from studies involving monoclonal antibodies (MAbs) and neutralization-resistant variants (Heidner *et al.* 1990; White & Eaton 1990; DeMaula *et al.* 1993; Hwang & Li 1993). These epitopes have been described as being conformationally dependent interactive sites within a single antigenic domain. Furthermore, it has been recognised that neutralization epitopes are not unique to each serotype (Huismans & Bremer 1981; Gould *et al.* 1988) but may exist on other serotypes in a neutralizing or non-neutralizing conformation such that the neutralizing domain of each serotype contains many distinct epitopes which vary in their significance to the neutralization of individual viruses (Ristow *et al.* 1988; White & Eaton 1990; Rossitto & MacLachlan 1992). The expression of

epitopes in a conformation that results in virus neutralization could be influenced by a variety of complex interactions between individual viral proteins, including that of VP2 with itself, as well as the interactions of VP2 with outer capsid protein VP5 and core proteins such as VP7.

The variability and serotype-specificity of segment 2 of AHSV was originally reported by Bremer *et al.* (1990) following hybridization studies on the genetic relatedness of a number of cognate genes within the AHSV serogroup. This data was recently corroborated by Laviada *et al.* (1993) in radioimmunoprecipitation and Western immunoblotting assays. Burrage *et al.* (1993) demonstrated that neutralizing epitope(s) for AHSV are located on VP2 by the isolation of VP2-specific MAbs capable of serotype-specific viral neutralization. Laviada *et al.* (1993) also made the observation that the most immunogenic epitope(s) on AHSV VP2 seem to be conformational, based on the weak recognition of the denatured protein in immunoblotting.

VP2 is, therefore, the main focus of research aimed at development of recombinant vaccines for AHSV.

In contrast to VP2, very little is known about the function of VP5. Although the protein is located in the outer capsid, antisera raised against baculovirus-expressed BTV VP5 do not demonstrate any neutralizing activity *in vitro* (Marshall & Roy 1990). However, there are indications that BTV VP5 contributes to virus neutralization (Cowley & Gorman 1989; Mertens *et al.* 1989; Roy *et al.* 1990) and it is generally assumed that VP5 enhances the immune response indirectly by interaction with VP2, thereby affecting the conformation of VP2 and, consequently, its serological properties (Roy 1992).

Although VP7 is part of the core particle, a BTV serogroup-reactive epitope on VP7 has recently been shown to be accessible on the surface of BTV particles (Lewis & Grubman 1990; Eaton *et al.* 1991). The fourth capsid protein, VP3, plays an important role in the structural integrity of the virus core, forming the protein scaffold of the subcore particle.

1.3 VACCINE DEVELOPMENT

Vaccination against infectious viral diseases through the elicitation of a protective immune response without causing clinical disease has met with considerable success in the history of veterinary medicine. This success has been achieved despite the complexity of aetiological organisms in terms of antigenic variability (Murray 1987). Protection against viral disease through vaccination can be accomplished by using a live attenuated virus vaccine, an inactivated virus, virus subunits either derived from infectious material or produced by genetic engineering involving specific gene expression, or synthetic peptides representing neutralization

epitopes (Roy *et al.* 1990).

1.3.1 Modified virus vaccines

Live attenuated virus vaccines

Current procedures for vaccination often involve the use of modified live virus vaccines, whether a naturally occurring variant of the virulent organism, or a virus artificially attenuated by laboratory manipulations, or inactivated virus vaccines. Such vaccines have been instrumental in the control of notifiable viral diseases, such as the diseases caused by foot and mouth disease virus (FMDV; Brown 1992), rinderpest virus (RPV; Bassiri *et al.* 1993) and bovine herpes virus (BHV-1; Van Drunen Littel-van den Hurk 1993; Yancey 1993). However, although effective, the use of these vaccines is limited because of the high cost of production and their heat lability. Live attenuated rabies virus has also been used with success for the oral vaccination of feral animals (Baer 1988). However, vaccines for such purposes have the additional prerequisite of being avirulent for both the target and for non-target species that may be occassionally infected. Recently, strains of rabies virus with several mutations that affect virulence have been prepared in an attempt to reduce the frequency of reversion to a pathogenic phenotype (Tidke *et al.* 1987). A new generation modified live vaccine for BHV-1 has been developed by modification of the thymidine kinase gene, which is associated with virulence, resulting in a stable, irreversible attenuating mutation in the gene (Yancey 1993).

Rotaviruses have a genome consisting of 11 segments of dsRNA, each coding for a viral protein. Of particular interest for vaccine development are the two outer capsid proteins, VP7, a glycoprotein on the viral surface, and VP4, which are important in virus neutralization and protection from disease. Initial efforts to develop a human rotavirus vaccine adopted a 'Jennerian' approach, i.e. the use of a live animal (bovine or simian) rotavirus, antigenically related to human rotavirus but naturally attenuated for humans, to immunize against the human disease (Vesikari *et al.* 1984). These vaccines were found to have variable protective efficacy in numerous field trials in a variety of settings, but failed to protect young children in developing countries (Glass *et al.* 1994). Live oral candidate vaccines which are currently being developed and tested are based on reassortant animal viruses which contain the VP7 gene of human rotaviruses incorporated into an animal parent strain (Glass *et al.* 1994). These could further be improved by the addition of a VP4 gene from a human strain as a separate reassortant, based on the theoretical advantage that neutralizing antibody to the other outer capsid protein (VP4) may give more comprehensive immunity than is possible with vaccines containing VP4 of animal rotavirus strains. As an alternative, human rotavirus strains that have

been attenuated by cold adaptation, multiple passaging, or selection of neonatal non-pathogenic isolates are also being investigated (Bishop 1993).

At present, both bluetongue (BT) and AHS are controlled predominantly by annual vaccinations with polyvalent live attenuated vaccines. The AHSV vaccine strains are attenuated by serial passage in Vero cell cultures. The vaccine currently in use in South Africa contains 8 distinct serotypes, administered as 2 quadrivalent doses. The doses are administered 28 days apart and precautions are advised to minimize exposure and stress (House *et al.* 1992a). Sheep are similarly vaccinated annually against BT with three pentavalent live attenuated vaccines administered successively at three-weekly intervals.

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.* 1987a, b; Stott *et al.* 1987; Katz *et al.* 1990). 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 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).

Inactivated virus vaccines

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. However, production of inactivated vaccines is complex and expensive and inactivated virus vaccines do not confer long-lasting immunity and thus require multiple doses. In addition, the afforded protection is not always complete and cross-protection of immunized animals is restricted to viruses of the same serotype (Kit *et al.* 1991). In the case of RPV, inactivated whole virus vaccines failed to protectively immunize cattle against rinderpest. Similar observations have been made for other members of the morbillivirus group (Norrby *et al.* 1975). In the case of foot and mouth disease (FMD), many of the outbreaks in the past have reportedly been caused by escape of virus from vaccine production units, or the use of

improperly inactivated vaccines (Beck & Strohmaier 1987). 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; Bahnemann 1990; Moormann *et al.* 1990; House *et al.* 1992b). Another major drawback of inactivated vaccines is the hypersensitization that has been reported when sheep previously vaccinated with inactivated BTV are challenged with virulent virus (Stott *et al.* 1985). No inactivated AHS or BT vaccines have as yet progressed to field trails (Campbell 1985; Stott *et al.* 1985).

1.3.2 Biosynthetic 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. 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 immunization.

By definition, the development of biosynthetic subunit vaccines involves the expression of non-infectious protective subunit immunogens through a biological system. In general, the neutralization specificity of antigens often appears to demand particular vectors (usually eukaryotic) for the expression of immunogens (Brown 1992; Bishop 1993). The absence of glycosylation and/or the requirement of virus structural constraints which fold the protein and form important conformational epitopes often results in poor neutralization activity of prokaryote-expressed proteins, although protection against FMD has reportedly been obtained by inoculation of cattle and swine with extracts from *Escherichia coli* expressing FMDV capsid proteins (Kleid *et al.* 1981; Grubman *et al.* 1993). The expression systems most extensively used for the expression of viral proteins include baculoviruses (Miller 1988) and vaccinia virus (Mackett & Smith 1986). Unfortunately, the limited immunogenicity of many subunit vaccine candidates has hindered their development as potential vaccines. Strategies to enhance their immunogenicity, for example by changing the conformation of the antigen, thereby enhancing antigenic presentation, or by inducing the production of various immunomodulatory cytokines that act directly on helper T (T_H) lymphocytes to stimulate specific types of immune responses, are therefore critical (Rabinovich *et al.* 1994).

Baculovirus subunit vaccines

Baculovirus-expressed viral proteins, particularly the capsid proteins, have been

demonstrated to prime for the development of antibodies that neutralize virus infectivity for, amongst others, BTV (Inumaru & Roy 1987; Roy *et al.* 1990, 1992), rotavirus (Redmond *et al.* 1993), FMDV (Grubman *et al.* 1993), rabies virus (Fu *et al.* 1993) and a rabies related virus, Mokola virus (Tordo *et al.* 1993). In contrast, however, vaccination of cattle with baculovirus-expressed RPV fusion and hemagglutinin glycoproteins failed to protect despite the presence of serum neutralizing antibodies (Bassiri *et al.* 1993).

In the case of BTV, vaccination of sheep 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). In conjunction with VP5, VP2 was found to elicit a higher titre of neutralizing antibodies and the protective immune response was enhanced. This could be attributed directly to VP5 or indirectly by interaction with VP2, affecting the conformation and consequently the serological properties of VP2.

Immunological studies of BTV infection have indicated that protection from reinfection involves components of both the humoral and cellular immune response (reviewed by Jeggo & Wardley 1985). It was found that the humoral response was type-specific whilst the cellular immune response, particularly through the action of cross-reactive cytotoxic T lymphocytes (CTLs), gave rise to heterotypic protection. While antigen preparations devoid of viral nucleic acid would be expected to stimulate the production of and react with neutralizing antibody, it is important to question whether such vaccines would also elicit a sustained T lymphocyte response of sufficient magnitude to prevent primary infection or temper the severity of recrudecent disease.

BTV CLPs have been demonstrated to be formed by the coexpression of VP3 and VP7 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 immunization with CLPs did not induce neutralizing antibodies, nor did it affect the neutralizing antibody response to the challenge virus (French *et al.* 1990). The finding that CLPs can induce protection in the absence of neutralizing antibodies suggested the involvement of a cell-mediated response, but this remains to be investigated (Van Dijk 1993).

Through coexpression of BTV VP2, VP3, VP5 and VP7 in recombinant baculovirus-infected cells, VLPs which appear to be similar to authentic virus particles in morphology and biochemical constitution assembled spontaneously (French *et al.* 1990). BTV VLPs were investigated for their vaccine potential in sheep and proved to be highly immunogenic (Roy *et al.* 1992). Adjuvants significantly enhanced the immune response and the degree of protection, implying once again that cell-mediated immunity, and T_H cells in particular, might

well play a major role in protection. 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). This could be ascribed to presentation of VP2 in its authentic conformation on the VLPs, or to an individual or combined effect of the other components of VLPs viz. VP3, VP5, VP7 to the protective humoral response and/or to cell-mediated immunity.

On the basis of these results, it is envisaged that a similar approach could be utilized in the development of an efficacious AHSV vaccine.

Rotaviruses similarly possess two capsid layers. The outer capsid is composed of two proteins, VP4 and VP7, which both independently evoke antibodies that neutralize virus *in vitro* and protect against rotavirus-induced disease *in vivo* (Offit & Blavat 1986; Offit *et al.* 1986a, b; Hoshino *et al.* 1988; Matsui *et al.* 1989). VP6 forms the major component of the inner capsid which is composed of four proteins, VP1, VP2, VP3 and VP6. Redmond *et al.* (1993) have cloned and expressed combinations of structural proteins VP4, VP6 and VP7 in a baculovirus system and investigated the capacity of the recombinant proteins and assembled particles to induce protective immunity in the murine model. Only antisera from animals immunized with preparations containing VP4 neutralized virus. Challenge of neonates born to animals immunized with VP4 protein on assembled particles or in cell lysates showed protection against challenge with both homologous and heterologous strains of rotavirus. In contrast, offspring of mice immunized with VP6 or VP7 were only partially protected. However, neonates of animals immunized with VLPs composed of VP7 assembled on VP6 spherical particles were protected against challenge with a homotypic strain and significantly protected from a heterotypic challenge. The reduction in clinical disease achieved with VP6 and VP7, as well as with double-shelled particles composed of VP6 and VP7, supports the importance of cell-mediated immunity in protection against rotavirus. In studies of the CTL response to rotavirus proteins, Dharakul *et al.* (1991) found that $CD8^+$ lymphocytes isolated from Balb/c mice immunized with baculovirus-expressed rotavirus VP1, VP4, VP6 and VP7 were all able to clear rotavirus infection in chronically ill severe combined immunodeficient (SCID) mice, whereas donated $CD8^+$ from mice immunized with VP2 and four nonstructural proteins were unable to.

Bachmann *et al.* (1994) recently evaluated the ability of non-infectious and non-replicating viral protein antigens derived from a baculovirus system to induce CTLs. The viral proteins were demonstrated to trigger long-lasting $CD8^+$ T cell-mediated antiviral immunity. The CTLs lysed infected target cells *in vivo* and protected mice from viral replication and immunopathological disease. This data is of obvious practical importance and should help to design vaccines inducing a balanced immunity, comprising B cells, T_H cells and CTLs.

Live viral vectors

An alternative approach to the development of recombinant antigens as vaccines is the use of live viral vectors, involving the generation of recombinant (particularly pox) viruses through the insertion of foreign genes, without impairing infectivity (reviews by Esposito & Murphy 1989; Cox *et al.* 1992). Such vaccines are capable of eliciting both humoral and cellular immunity, and the persistence of antigen expressed by recombinant virus-infected cells provides a vigorous stimulation of the immune system. Such vaccines may thus provide lifelong immunity with a single dose, without any need for the addition of carriers, activators or adjuvants.

Several features of vaccinia virus, including its ability to tolerate insertions of large segments of foreign DNA as well as its replication within the cytoplasm of host cells, makes this virus an ideal vector for expression of antigens from a large range of pathogens (Cooney *et al.* 1991). Recombinant vaccinia virus vaccines have been investigated, and reported to induce protective immunity, for a number of human and veterinary diseases including herpes simplex virus (HSV; Weir *et al.* 1989), equine herpes virus (EHV; Guo *et al.* 1989), measles virus (Drillien *et al.* 1988), RPV (Yilma *et al.* 1988; Belsham *et al.* 1989; Giavedoni *et al.* 1991), rotavirus (Andrew *et al.* 1992) and rabies virus (Kieny *et al.* 1984; Wiktor *et al.* 1984; Rupprecht *et al.* 1988; 1992). In the case of rinderpest, cattle vaccinated with recombinant vaccinia viruses expressing the F and/or H glycoproteins of RPV were found to be completely protected against lethal RPV challenge. As noted earlier, however, cattle vaccinated with baculovirus-expressed RPV F and H glycoproteins were not protected from rinderpest, despite the presence of serum neutralization antibody. Similar observations have previously been made with inactivated whole virus vaccines. This provided indirect evidence that cell-mediated immunity may play an essential role in protection against rinderpest (Bassiri *et al.* 1993).

Although the critical determinants of the immune response associated with protection against rotavirus infection are unknown, there are several studies which support a hypothesis that rotavirus-specific CTLs may be important and in part explain the heterotypic protection found after rotavirus immunization (Offit & Dudzik 1988, 1990; Offit & Svoboda 1989; Dharakul *et al.* 1990). Using vaccinia virus recombinants expressing individual rotavirus proteins, Offit *et al.* (1994) found that only recombinants expressing VP7 evoked detectable rotavirus-CTLs which lysed target cells infected with different rotavirus serotypes whereas recombinants expressing VP1, VP4 and VP6 failed to induce a detectable CTL response. This is in contrast to the earlier findings of Dharakul *et al.* (1990) with baculovirus-expressed rotavirus proteins. According to Offit *et al.* (1994) this may be related to the manner in which exogenous proteins and endogenously synthesized proteins are presented to the immune

system.

However, since efficacy and safety are both very important criteria for evaluation of potential vaccines, the relatively low immunogenicity of expressed antigens and the adverse clinical reactions reported in response to wild type vaccinia virus (Lane & Millar 1971) reduces its potential as a suitable vector. Furthermore, the broad host range of vaccinia virus poses the risk of spread to non-target species. Extensive use of vaccinia based recombinant vaccines may lead to a wide dissemination of vaccinia with the possibility of recombination occurring with other orthopoxviruses present in non-target animals.

Other poxviruses with much more restricted host ranges have been considered for use as a basis for recombinant vaccines, for example canarypox virus (Taylor *et al.* 1991), fowlpox virus (Boyle & Coupar 1988; Taylor *et al.* 1988a, b, 1990; Boursnell *et al.* 1990; Ogawa *et al.* 1990; Nazerian *et al.* 1992; Heine & Boyle 1993), capripoxvirus (Romero *et al.* 1993) and raccoon pox virus (Esposito *et al.* 1988). Capripoxvirus isolates are already in use as effective vaccines against sheep and goat pox and lumpy skin disease of cattle, and recently the development of a capripoxvirus-based recombinant vaccine which expresses the F protein of RPV under the control of the vaccinia virus late promoter p11, was reported. This vaccine was found to protect cattle against a lethal challenge with virulent rinderpest and against lumpy skin disease (Romero *et al.* 1993). Besides poxvirus-based vectors, several other virus families, such as *Adenoviridae* (Eloit *et al.* 1990) and *Herpesviridae* (Kit *et al.* 1991) etc., have been reported as candidates for use as viral vectors in the vaccination of animals. Eloit *et al.* (1990) have described the construction of a defective adenovirus vector, expressing the pseudorabies virus (PRV) glycoprotein gp50, which yielded partial protection against PRV in rabbits and mice. The authors anticipated that the defect would lower the levels of replication *in vivo* and thus limit the risks of dissemination of recombinant virus despite the wide host range.

Peptide vaccines

Another approach to subunit vaccine development involves the chemical synthesis of peptides which represent immunogenic domains of a particular virus antigen (Shinnick *et al.* 1983). The simplest approach is to link B cell and T cell (T_H and CTLs) epitopes and use these linear polyepitopes as vaccines. In principle, these strategies result in an immune response directed only to the relevant epitope(s) on the pathogen, and therefore may avoid any toxicity associated with an immune reponse to other epitopes. As a result, peptide vaccines are anticipated to be better defined, more stable and safer than other vaccines, requiring no infectious agent. Furthermore, they can be designed to stimulate the desired immune response and, provided adequate delayed release mechanisms can be designed, booster doses could be

delivered from implants (Brown 1992).

In practice, epitope-based approaches may stimulate good antibody responses but not potent cellular immunity, especially CTL responses (Rabinovich *et al.* 1994). Thus, although synthetic peptides are currently less effective than natural viral proteins in eliciting immune responses and are expensive to produce, they may become more feasible as B cell, T_H and CTL epitopes are elucidated and their sequences linked. Peptide vaccines have been successfully employed in conferring protection against such diseases as influenza, serum hepatitis and FMD (Dix 1987).

A prerequisite for this approach is a precise knowledge of the identity and structure of virus-neutralizing epitopes. Several empirical methods to determine the presumed location of continuous B cell epitopes in native proteins, that are based on the properties of the amino acid residues, have been developed (reviewed by Leinikki *et al.* 1993). These include hydrophilicity / hydrophobicity, segmental amphipathicity / amphiphilicity, flexibility, surface exposure and sequence variability. In the case of FMD, several of these approaches were utilized independently for the selection of peptides for analysis of their ability to elicit neutralizing antibodies (Bittle *et al.* 1982; Pfaff *et al.* 1982; Strohmaier *et al.* 1982; Geysen *et al.* 1984). The VP1 regions 141-160, which is located in the middle of a highly variable region exposed on intact virus (Robertson *et al.* 1983) and 200-213, which is located close to the former region, were identified as epitopes for neutralizing antibodies. Subsequently, synthetic peptides based on 141-160, with or without carrier proteins, have been used to raise antibodies in mice, rabbits and guinea pigs and to protect the latter from challenge against homologous virus (Francis *et al.* 1985, 1987; Murdin & Doel 1987). Furthermore, the immunogenicity of this peptide was increased by presentation of the peptide fused with the hepatitis B core protein, which spontaneously assembles into particles with multiple copies of the peptide repeated over the surface (Clarke *et al.* 1987). However, the natural extension of these results to protect the major target species i.e. cattle, has only been reported by DiMarchi *et al.* (1986) using a synthetic peptide that contained residues 141-158 and 200-213.

Dietzschold *et al.* (1990) utilized the deduced amino acid sequences of neutralization-resistant variant rabies viruses to map a linear epitope of the rabies virus glycoprotein. A tandem peptide constructed from this epitope and a dominant T_H cell epitope of the nucleoprotein induced protective immunity against lethal rabies virus challenge infection in mice.

Kit *et al.* (1991) reported a new approach to biosynthetic FMD vaccines which utilizes a modified-live BHV-1 (or infectious bovine rhinotracheitis virus, IBRV) as a vector to express FMDV VP1 epitope sequences (amino acids 200-213 and 141-158) as part of a fusion protein with IBRV glycoprotein gIII. The FMDV epitopes were demonstrated to be presented as

repeated structures on the surface of the recombinant virus particles. Furthermore, the recombinant IBRV-FMDV viruses protected cattle from IBRV challenge and induced protective levels of FMDV neutralizing antibodies in cattle.

As briefly discussed earlier, considerable effort has been extended in the identification of possible neutralization domains on VP2 of BTV, which could serve in the production of immunogenic peptides for testing as potential BTV subunit vaccines. However, there is a distinct shortage of conclusive results in this regard, due largely to the complexity and extensity of interserotypic variation. In the case of AHSV, however, no investigations into possible epitopes on VP2 have as yet been carried out due to the lack of cloned and characterized VP2 gene sequences available.

Nucleic acid vaccines

A novel approach for the induction of immune responses has recently been described, opening a new avenue for vaccine development. Injection of plasmid DNA encoding an antigen of interest into muscle was shown to result in sustained expression of the antigen and generation of an immune response (Wolff *et al.* 1990; Tang *et al.* 1992). These vaccines appeared to stimulate persistent humoral and cell-mediated immune responses, without integration of plasmid into chromosomal DNA (Wolff *et al.* 1992). Ulmer *et al.* (1993) reported that immunization of mice with a plasmid carrying the influenza virus nucleoprotein gene under a mammalian promoter led to transfection of muscle cells, followed by transient expression of the protein, resulting in an influenza-specific immune response and consequently in protection against viral challenge. Xiang *et al.* (1994) subsequently demonstrated that immunization of mice with a mammalian expression vector carrying the rabies virus glycoprotein gene under the control of an SV40 early promoter induced a full spectrum of rabies virus glycoprotein-specific T and B cell mediated immune responses, and subsequently full protection against viral challenge. Although numerous questions remain about the possible mechanisms involved in vector vaccination, this technique has several advantages, including the ease of cloning of viral genes into expression vectors which can then be expanded and purified within days. This technique is potentially useful in protecting against viral challenge in which the antibody response alone is not protective, or where there is antigenic diversity of surface proteins among strains (Rabinovich *et al.* 1994). This strategy results in relevant antigen production in the host without the use of infectious agents, whereas, besides the time consuming and labour intensive efforts of cloning genes into heterologous eukaryotic expression systems and purification of proteins, the use of recombinant pathogens may result in a potent response against the carrier which might bias the response against the insert (Xiang

et al. 1994).

1.4 CONCLUSION AND AIMS

As can be deduced from the aforegoing, there are numerous approaches to the development of viral vaccines through the application of modern recombinant DNA technology, creating a menu of options that require rational evaluation. The application of these techniques and the efficacy of a vaccine against a particular viral disease will require careful investigation and evaluation on individual merit.

Bishop (1993) concluded that the strategic stages in the development of any candidate vaccine are to define the immunological mechanisms of protection after natural infection; to identify the antigens that induce this protection; to select live attenuated organisms that bear these antigens or utilize molecular biological techniques to produce these antigens; to optimize vaccine composition and delivery; and, finally, to test for safety and protective efficacy.

As discussed, the current AHS vaccine, although effective, does involve certain risks and 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. As described in the preceding review, a number of possible approaches demand investigation. However, in the development of any recombinant vaccine, it is mandatory to identify and characterize all the antigens required to stimulate a protective immune response. In order to achieve this, the antigen encoding genes have to be cloned and characterized, and sufficient antigen has to be isolated for immunogenic analysis.

Accordingly, in the pursuit of a producer- and user-friendly vaccine for AHSV, and based on the available information in literature, the goal of this study was to investigate the outer capsid protein, VP2, of AHSV as the main determinant of serotype specificity and the neutralization-specific immune response. In order to accomplish this, the following aims were envisaged:

- Cloning of a full-length copy of the AHSV-3 VP2 gene, genome segment 2.
- Characterization of the AHSV-3 VP2 gene by sequence determination.
- Expression of AHSV-3 VP2 protein in a eukaryotic expression system.

CHAPTER 2

CLONING AND SEQUENCING OF GENOME SEGMENT 2 OF AFRICAN HORSESICKNESS VIRUS SEROTYPE 3

2.1 INTRODUCTION

VP2 is a major structural protein of AHSV, forming the outer capsid layer of the virion. VP2 of orbiviruses has been shown to be the most variable of the viral proteins (Huismans *et al.* 1987a; Bremer *et al.* 1990; Iwata *et al.* 1992). It has been demonstrated that neutralizing epitopes for AHSV are located on VP2 and, as such, may play a role in the determination of serotype-specificity and the neutralization-specific immune response (Burrage *et al.* 1993). However, little is known of the identity and location of these epitopes.

In order to investigate the serotype-specific nature of VP2, and to gain an understanding of the structure of the protein, it is necessary to obtain more information regarding the nucleotide sequence of the gene that encodes VP2. Knowledge of the amino acid sequence of VP2 could allow the identification and characterization of possible functional domains or epitopes, and predictions about the secondary and tertiary structure could be made.

The nucleotide sequences of orbivirus VP2 encoding genes which have been published include those of BTV-1 (Gould & Pritchard 1990), BTV-2 (Yamaguchi *et al.* 1988), BTV-3 (Gould & Pritchard 1990), BTV-10 (Purdy *et al.* 1985), BTV-11 (Ghiasi *et al.* 1987), BTV-13 (Fukusho *et al.* 1987), BTV-17 (Ghiasi *et al.* 1987), epizootic haemorrhagic disease virus (EHDV-1; Roy *et al.* 1992) and AHSV-4 (Iwata *et al.* 1992). Numerous comparisons between the various known BTV VP2 sequences have been made in order to investigate the relatedness of the different serotypes and to identify possible antigenic determinants (Fukusho *et al.* 1987; Ghiasi *et al.* 1987; Gould 1988; Yamaguchi *et al.* 1988). The relatedness between the capsid protein genes of AHSV, BTV and EHDV has also been investigated (Iwata *et al.* 1992).

If the nucleotide sequence of another AHSV VP2 gene were known, comparisons with the AHSV-4 VP2 gene sequence could be made to determine the variability and phylogeny between different AHSV serotypes and to study the molecular epidemiology. Furthermore, analysis of the patterns of interserotype variability of VP2 in different orbiviruses would enable the identification of conserved and variable orbiviral VP2 features, and as such may provide

more insight into the identity and location of various epitopes involved in the host immune response.

In this chapter, the strategy of cloning and sequencing the AHSV-3 VP2 coding gene is described, as well as comparisons between the nucleic acid and amino acid sequences of VP2 coding genes of various orbiviruses. Although it is possible to directly sequence the genomic dsRNA of AHSV, it was decided to synthesize and clone cDNA to facilitate the sequencing of the segment 2 gene. The main advantages thereof are the subsequent simple isolation and purification of large quantities of the cloned gene, and the many standard molecular biological manipulations to which it can be subjected, including subcloning and expression in different host systems.

2.2 MATERIALS AND METHODS

2.2.1 Cells and viruses

A South African isolate of African horsesickness virus type 3 (AHSV-3), obtained from the OVI and described by Bremer *et al.* (1990), was used in this investigation. The virus was propagated by limited passaging in baby hamster kidney cells (BHK-21 originally obtained from the American type culture collection (ATCC), Maryland, USA) grown as monolayers in Roux flasks or roller bottles as described by Huismans (1979) using modified Eagle's medium supplemented with 5% bovine serum (Verwoerd 1969).

2.2.2 Isolation and purification of viral dsRNA

AHSV-3 dsRNA was isolated from infected cells by the SDS-phenol extraction method described by Huismans & Bremer (1981). Monolayers of BHK-21 cells infected with AHSV-3 at a multiplicity of infection (MOI) of 10 plaque forming units (pfu)/cell were harvested at 48 h post infection (pi) by low speed centrifugation and resuspended in 80ml 2mM Tris pH9.0. Sodium acetate (NaAc) pH5.0 and ethylenediaminetetra-acetic acid (EDTA) were added to final concentrations of 10mM each and then sodium dodecyl sulfate (SDS) was added to a final concentration of 1% (m/v). The pH of the solution was adjusted to 5.0 with glacial acetic acid before extracting the solution twice with 0.5 volume of phenol at 60°C (15 min 60°C, 15 min on ice, 15 min centrifugation at 10000g). Phenol residues were removed with two chloroform extractions and the RNA precipitated by the addition of 0.1M NaCl and two volumes ethanol. The precipitate was dissolved in 0.01M STE (10mM NaCl, 10mM Tris-HCl pH7.6, 1mM EDTA) and the ssRNA was removed by salt precipitation in 2M LiCl. The supernatant was then diluted with 0.01M STE to 0.2M LiCl and the dsRNA was purified by methylated albumin Kieselguhr (MAK) column chromatography (Osawa & Sibatani 1967).

MAK column chromatography involved the following: 15g of Hyflo-supercel (BDH chemicals) was suspended in 75ml 0.2M saline buffer (0.2M NaCl, 50mM Tris pH6.7) and boiled for 1 min to expel air in the supercel. After cooling, 4ml of a 1.5% (m/v) aqueous solution of methyl albumin was added slowly while stirring. A 1.5 x 30cm column was packed with the material and rinsed with 70ml 0.2M

saline buffer using a peristaltic pump. The sample was loaded onto the column at a salt concentration of approximately 0.2M and passed into the column at a speed of approximately 1ml/min. Elution was effected with a linear concentration gradient of 0.2M to 1.2M saline buffer. 80 drops/fraction were collected with an LKB fraction collector, the dsRNA normally eluting at fractions 25 to 35 (approximately 0.6M NaCl) as determined by agarose gel electrophoresis. The dsRNA was precipitated by the addition of two volumes ethanol and collected by centrifugation.

Thereafter, the total dsRNA was fractionated by sucrose gradient centrifugation. The sample was loaded onto a 5ml gradient of 5-40% sucrose in 1x TE buffer (10mM Tris pH7.4, 1mM EDTA pH8.0) and centrifuged for 16 h at 38000 rpm in a Beckman SW50.1 rotor at 4°C. Gradients were fractionated using a gradient tube fractionator (Hoefer Scientific Instruments) and collecting 8-10 drops/fraction. A small sample of each fraction was analysed by 0.8% agarose gel electrophoresis and fractions containing predominantly large-, medium- or small-sized genome segments were pooled. The pooled fractions were diluted in an equal volume of water, NaAc pH8.0 was added to a final concentration of 0.3M and the dsRNA ethanol precipitated.

2.2.3 Cloning of dsRNA

Polyadenylation of dsRNA

Preparations of AHSV dsRNA containing approximately 10-50 µg predominantly large genome segments were originally denatured with 10mM methyl mercuric hydroxide (MMOH) for 10-15 min at room temperature (RT). However, this denaturation step was subsequently omitted. The RNA was then polyadenylated (Sippel 1973) by incubation at 37°C for 15 min with 6 units of *Escherichia coli* poly A polymerase (Gibco BRL) in the presence of 50mM Tris-HCl pH8.0, 10mM $MgCl_2$, 2.5mM $MnCl_2$, 250mM NaCl, 0.25mM ATP and 10 µCi ^{3}H-ATP (49Ci/mmol, Amersham) in a final volume of 300 µl. The reaction was terminated by the addition of EDTA to a final concentration of 8mM and loaded onto a Sephadex G-75 column equilibrated with 1mM Tris-HCl pH8.0. A 2µl sample from each 100 µl fraction was counted in liquid scintillation fluid. The fractions represented by the first peak were pooled and lyophilized in a siliconized glass tube.

Sephadex column chromatography

Sephadex G-75 (Sigma) beads with a molecular weight fractionation range of 3000-80000 (globular proteins) or 1000-50000 (dextrans) were swollen overnight in 1x TE buffer and sterilized by autoclaving. A column was packed in a 5ml Pasteur pipette blocked with a small glass bead and rinsed with 3ml of an appropriate buffer. The sample to be purified was layered onto the column in a buffer volume of 100 µl and rinsed into the column with 600 µl buffer. Thereafter, the sample was eluted with buffer in 12 100 µl fractions which were counted in a liquid scintillation counter. The labelled nucleic acid was represented by an initial peak followed by a second peak of unincorporated free nucleotides.

Synthesis and size fractionation of cDNA

Approximately 0.5-3 µg polyadenylated RNA was denatured in 20mM MMOH for 15 min at RT. cDNA synthesis was carried out at 42°C for 1 h by the addition of 1µg oligo $(dT)_{15}$ (Boehringer Mannheim) and 18 units avian myeloblastosis virus (AMV) reverse transcriptase (Promega) in the presence of 50mM Tris-HCl pH8.3, 10mM $MgCl_2$, 70mM KCl, 3mM 2-mercaptoethanol, 100 units human placental ribonuclease inhibitor (Amersham), 0.5mM each dNTP and 20 µCi α^{32}P-dCTP

(>400Ci/mmol, Amersham) in a final volume of 60µl. The reaction was stopped by the addition of EDTA and diluted to a final volume of 100µl with 1mM Tris-HCl pH8.0. The cDNA was separated from unincorporated nucleotides by Sephadex G-75 column chromatography in 1mM Tris pH8.0 and the α^{32}P-dCTP incorporation was monitored by Cerenkov counting. The cDNA fractions were pooled and lyophilized. The reaction was scaled up as required.

Lyophilized cDNA was incubated in a suitable volume of 10x alkaline buffer (0.3M NaOH, 20mM EDTA) for 30 min at RT to hydrolyse the RNA and then either separated by vertical 2% agarose gel electrophoresis in 1x alkaline buffer, in which case the wet gel was covered in Glad wrap and autoradiographed overnight prior to excision of gel slices containing large, medium and small cDNA genome segments respectively, or layered onto a handpacked linear density gradient consisting of 10-40% sucrose in 1x alkaline buffer. The gradient was centrifuged at 48000 rpm for 16 h at 4°C in a Beckman SW50.1 rotor and fractionated by drop collection using a gradient tube fractionator (Hoefer Scientific Instruments). The fractions were analysed by Cerenkov counting and three pools of fractions in the rising slope of the peak were collected. Pool samples were analysed by vertical 2% agarose gel electrophoresis in 1x alkaline buffer and autoradiography of the dried gels to determine the segment representation of each pool.

The addition of $^1/_5$ volume of 100x TE to stabilize the pH preceded the recovery of the cDNA from the gel slices or sucrose pools by Geneclean™ II kit methodology (2.2.8).

C-tailing of cDNA and annealing in pBR322

Homopolymeric dC tails were added to the 3' ends of the cDNA as described by Deng & Wu (1981) using 15 units terminal deoxynucleotidyl transferase (TdT, Gibco BRL) in the presence of 0.1mM dCTP and 20µCi α^{32}P dCTP (>400Ci/mmol, Amersham) in $^1/_5$ volume 5x DNA tailing buffer (0.5M potassium cacodylate pH7.2, 10mM $CoCl_2$, 1mM dithiothreitol (DTT)). The reaction was incubated at 37°C for 15 min and the cDNA was purified by the Geneclean™ II kit methodology (2.2.8) or by NENSORB™ 20 (NEN Research Products) column chromatography. The latter column was first rinsed with 4ml 100% methanol and then with 2ml Reagent A (0.1M Tris-HCl, 10mM Triethylamine, 1mM EDTA, pH7.7). cDNA diluted to 1ml with 1x TE was loaded onto the column and forced through with gentle pressure from an air-filled syringe (approximately 1 drop per 2 seconds) to allow the cDNA to adsorb. After thorough rinsing of the column with 3ml Reagent A and then 1ml water, the cDNA was eluted with 50% methanol in 200µl fractions. The cDNA was typically recovered in the first 400µl and lyophilized in a siliconized glass tube. Cerenkov counting was used to monitor the purification procedure throughout.

The dC-tailed cDNA was incubated with 200-400ng dG-tailed PstI-cut pBR322 (Gibco BRL) in annealing buffer (10mM Tris-HCl pH8.0, 150mM NaCl, 2mM EDTA) for 5 min at 80°C and 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 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. *E. coli* HB101, JM105 or JM109 cells were routinely used for transformations with pBR322, pUC13 and M13mp19 DNA respectively. *E. coli* JM109 cells were maintained on glucose/minimal medium plates (1.5% agar, 40mM Na_2HPO_4, 20mM KH_2PO_4, 20mM NH_4Cl, 10mM NaCl, 10mM $MgSO_4$, 10mM thiamine HCl, 1mM $CaCl_2$, 0.2% glucose) to select for bacteria carrying the F' plasmid. An overnight

E. coli cell culture was used to inoculate 80ml Luria-Bertani (LB) medium (1% tryptone (m/v), 0.5% yeast (m/v), 1% NaCl (m/v)) and the cells were grown to logarithmic (log) phase (OD_{550} = 0.5) by incubation at 37°C with shaking. The cells were collected by centrifugation (5000 rpm, 5min, 4°C), gently washed in half the original volume of ice cold freshly prepared 50mM $CaCl_2$, collected by centrifugation and resuspended in $^1/_{20}$ of the original volume of $CaCl_2$. The cells were kept on ice for at least 1 h before transformation.

2.2.5 Transformation of competent cells with plasmid DNA

Plasmid DNA was added to 200µl of the appropriate competent cells and allowed to adsorb for 30 min on ice. The cells were then subjected to a heat-shock at 42°C for 90 s and cooled on ice for 2 min before having 0.8-1ml LB medium added and being incubated at 37°C with shaking for 1 h. Aliquots of 100 to 150µl were then plated out with 50µl 2% 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal) in dimethylformamide and 10µl 100mM isopropyl-ß-D-thiogalactopyranoside (IPTG) when relevant on LB agar plates (1.2% agar in LB medium) containing the appropriate antibiotic (12.5µg/ml tetracycline hydrochloride (tet) or 100µg/ml ampicillin (amp)). Plates were incubated overnight at 37°C. Colonies of the desired phenotype (when selectable) were picked with sterile toothpicks and grown overnight in 5ml LB medium with the appropriate antibiotic.

2.2.6 Transfection of competent cells with bacteriophage M13 DNA

Following adsorption of bacteriophage M13 DNA to 200µl *E. coli* JM109 competent cells on ice for 30 min, a heat-shock treatment at 42°C was administered for 45 s followed by a 5 min incubation period on ice. Thereafter, 3 to 4ml molten soft agar (0.6% agar in LB medium) kept at 45°C and a mixture containing 200µl log phase *E. coli* JM109 cells, 40µl 2% X-gal in dimethylformamide and 40µl 100mM IPTG were added to the cells and poured onto an LB agar plate. The plates were incubated overnight at 37°C. Individual plaques of the required phenotype were picked as agar plugs and used to infect 5ml LB medium inoculated with 50µl fresh overnight culture *E. coli* JM109. The cultures were incubated with shaking at 37°C for 5 h.

2.2.7 DNA purification and characterization

dsDNA isolation and purification

Plasmid and M13 replicative form (RF) DNA extraction from liquid cultures of bacterial cells were done by the alkaline lysis method of Birnboim & Doly (1979) as described by Ausubel *et al.* (1988). A single bacterial colony was transferred to 5ml LB medium containing the appropriate antibiotic with a sterile toothpick and incubated overnight at 37°C. Cells in 3ml culture were harvested by bench top centrifugation for 2 min and resuspended in 100µl of an ice cold solution containing 25mM Tris-HCl pH8.0, 50mM glucose and 10mM EDTA. After 5 min on ice, 200µl of a freshly prepared solution of 0.2N NaOH, 1% SDS was added, mixed gently and incubated on ice for 5 min, lysing the bacteria and causing denaturation of the proteins as well as the chromosomal and plasmid DNA. 150µl 3M NaAc pH4.8 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 for 10 min and transferred to a fresh tube for ethanol precipitation of the plasmid DNA. The plasmid extraction was scaled up as required. Plasmid and M13

RF DNA were characterised by comparison to wild type controls in 0.8% agarose gel electrophoresis in 1x TAE (0.04M Tris-acetate, 1mM EDTA pH8.5) and by restriction endonuclease (RE) analysis (2.2.8). To obtain preparations free of contaminating RNA and protein, plasmid or M13 RF DNA was further purified by centrifugation to equilibrium in CsCl/ethidium bromide density gradients. 3.234g CsCl was dissolved in 3ml 1x TE containing the DNA before adding 300µl 10mg/ml ethidium bromide. The solution was centrifuged in a Beckman SW50.1 rotor at 38000 rpm for 40 h at 20°C. DNA bands were visualized under ultraviolet (UV) light and the lower band extracted by a syringe with a bent needle. The ethidium bromide was removed by extraction with water-saturated buthanol and the DNA was recovered by ethanol precipitation.

M13 ssDNA preparation and characterization

The supernatants of bacteriophage M13-infected cell cultures were collected by centrifugation and 20µl samples were taken for analysis of the single stranded (ss)DNA by 0.7% agarose gel electrophoresis in 0.5x TBE following the addition of 1µl 2% SDS and incubation at 65°C for 5 min. ssDNA of recombinant M13 bacteriophages suitable for sequencing was prepared as follows: bacteriophage particles were precipitated from the supernatant of infected cell cultures by the addition of $^1/_5$ volume of PEG/NaCl (20% polyethylene glycol 6000 (m/v), 2.5M NaCl). After 15 min at RT, the bacteriophage particles were recovered by centrifugation and resuspended in 1x TE. The bacteriophage M13 ssDNA was isolated by phenol extraction at RT, followed by two chloroform extractions of the aqueous phase to remove traces of phenol. The ssDNA was ethanol precipitated and resuspended in 1x TE.

2.2.8 Subcloning of restriction fragments

Restriction endonuclease digestion

RE digestions of plasmid or M13 RF DNA were carried out in the recommended salt buffer supplied with the enzyme (Amersham or Boehringer Mannheim) for 1 h at the temperature recommended for the enzyme concerned. Digestion products were analysed by 0.8% agarose gel electrophoresis and compared with DNA markers of known molecular weights.

Vector dephosphorylation

Dephosphorylation of linearised plasmid DNA was carried out by 3x dilution of the RE digestion reaction with $^1/_{10}$ volume 10x dephosphorylation buffer (0.5M Tris-HCl, 1mM EDTA, pH8.5) and water and the addition of 1 unit calf intestinal alkaline phosphatase (CIP, Boehringer Mannheim). The reaction was incubated at 37°C for 30 min.

Geneclean™ II or glasswool purification of DNA

For the construction of recombinant plasmids through cloning, DNA fragments of interest were cut out of agarose gels and isolated through Geneclean™ II kit (Bio101) methodology or by centrifuging (15000 rpm, 10 min) the gel slice through glass wool packed in an Eppendorf tube the base of which had been pierced with a needle. The DNA was then collected by ethanol precipitation.

The Geneclean™ II method involved the melting of the gel slice at 50°C in 2.5 volumes 6M NaI followed by the addition of 5-10µl of the glassmilk suspension. Glassmilk is a specially formulated

suspension of silica matrix that binds single- and double-stranded DNA without binding contaminants. The DNA was allowed to bind to the glassmilk by incubation on ice for 0.5-1 h with periodic agitation. After pelleting the glassmilk with the bound DNA, the pellet was washed three times with 0.5ml NEW wash (NaCl, Tris, EDTA, ethanol, water) prior to eluting the DNA twice with water at 45-55°C for 10 min.

Ligation

Ligations of restriction fragments of foreign DNA into suitable RE-linearised vectors was effected with 1 unit of T4 DNA ligase (Boehringer Mannheim) in ligation buffer (66mM Tris-HCl, 5mM $MgCl_2$, 1mM DTT, 1mM ATP, pH7.5) in a final volume of 15µl overnight at 15°C. Insert:vector molar ratios were approximated at 2:1. Half the ligation mixture was used to transform competent *E. coli* cells (2.2.4 - 2.2.6).

2.2.9 Genome segment assignments of recombinant plasmids

Labelling of probes by nick translation

Radioactive labelling of DNA was carried out by nick translation (Rigby *et al* 1977) using a commercially available kit (Promega). Nicks introduced in the DNA by DNase I are translated by a combination of the exonuclease and polymerase functions of DNA polymerase I, incorporating radioactively labelled nucleotides. 200ng DNA was incubated in a nick translation buffer (50mM Tris pH7.2, 10mM $MgSO_4$, 0.1mM DTT) with 20µM of each dATP, dGTP and dTTP, 10µCi α^{32}P-dCTP (>400Ci/mmol, Amersham), 1 unit DNA polymerase I and 0.2ng DNase I for 1 h at 15°C. The reaction was stopped by the addition of EDTA pH8.0 to a 25mM final concentration and loaded onto a Sephadex G-75 column equilibrated with 1x TE, 0.5% SDS. The specific activity of probes was generally in the order of 2 x 10^7 cpm/µg DNA.

Northern blotting of total dsRNA

The AHSV-3 dsRNA genome segments were separated by polyacrylamide gel electrophoresis (PAGE) using the buffer system described by Loening (1967). Preparative 6% acrylamide, 0.16% bisacrylamide gels were prepared by polymerization in Loening buffer (40mM Tris-HCl pH7.8, 20mM NaAc, 2mM EDTA) containing 0.08% ammonium peroxodisulfate (m/v) and 0.0008% N,N,N',N'-tetramethylethylendiamin (TEMED) (v/v) and electrophoresis was carried out at 80V for 22 h. After staining in ethidium bromide, the genome segments were visualized by UV fluorescence and their positions blueprinted. The gel was soaked in 0.1N NaOH for 30 min to denature the dsRNA and then washed in 0.5x TAE. The RNA was transferred to Hybond N (Amersham) nylon membrane by electroblotting with a trans blot cell (Biorad) in 0.5x TAE for 3 h at 0.8A and fixed to the membrane by UV exposure. The blueprint was used to pinpoint the positions of the genome segments on the membrane. Strips cut from the membrane were then used for hybridization with individual probes.

2.2.10 Hybridization

Membranes with fixed nucleic acids were prehybridized in hybridization buffer (0.75M NaCl, 0.5M $NaH_2PO_4.H_2O$, 5mM EDTA pH7.4, 50% deionised formamide, 0.1% fat-free milk powder, 0.2%

SDS) at 42°C for 30 min. Probes were denatured by the addition of $^1/_{10}$ volume of 1N NaOH and boiling for 5 min. The probe was then neutralized while on ice by the addition of $^1/_{10}$ volume of 1N HCl before being added to the membrane in hybridization buffer and left overnight at 42°C. The membrane was washed twice for 10 min in 2x SSC (0.3M NaCl, 30mM trisodium citrate; pH7.0) at RT, once in 2x SSC, 0.5% SDS at 37°C for 20 min and once in 2x SSC, 0.5% SDS at 65°C for 15 min before being autoradiographed overnight.

2.2.11 DNA sequencing

Sequencing was performed on CsCl-purified double-stranded plasmid DNA or phenol-purified single-stranded bacteriophage M13 DNA templates using the dideoxynucleotide method of Sanger *et al.* (1977). Reactions were carried out according to the protocols of the Sequenase™ Version II kit (USB). The Sequenase™ enzyme is a modified form of T7 DNA polymerase, its most important property being the absence of the 3'-5' exonuclease activity. dsDNA was first denatured in 0.2N NaOH, 0.2mM EDTA and ethanol precipitated after the addition of 0.25M Tris-HCl pH7.4 and 0.3M NaAc pH4.6. M13/pUC forward and reverse and pBR322-PstI (+) and (-) oligonucleotide primers as specified by Boehringer Mannheim, as well as synthetic oligonucleotides complementary to insert-specific sequence, were used for sequencing of recombinants (Table 2.1). 0.5pmol of the appropriate sequencing primer and 2µl 5x reaction buffer (200mM Tris-HCl pH7.5, 100mM $MgCl_2$, 250mM NaCl) were added to the dried denatured DNA in a final volume of 10µl and incubated at 37°C for 30 min. This was followed by the addition of 1µl 0.1M DTT, 2µl 1:5 diluted labelling mix (1.5µM of each of dCTP, dGTP and dTTP), 0.5µl ^{35}S dATP (Amersham, 1200Ci/mmol, 10mCi/ml) and 2µl Sequenase™ enzyme diluted 1:8 in dilution buffer (10mM Tris-HCl pH7.5, 5mM DTT, 0.5mg/ml bovine serum albumin (BSA)) and incubation at RT for 5 min. 4 x 3.5µl was then transferred to 4 tubes each containing 2.5µl termination mix (80µM of each dNTP, 50mM NaCl and 8µM of ddATP, ddCTP, ddGTP or ddTTP) and incubated at 37°C for 5 min. The reactions were terminated by the addition of 4µl stop solution (95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) and then heated to 85°C for 5 min prior to PAGE analysis on 370 x 300 x 0.4mm denaturing gels containing 6% acrylamide (m/v), 0.3% bisacrylamide (m/v) and 7M urea in 1x TBE (90mM Tris-HCl pH8.3, 90mM boric acid, 2.5mM EDTA). Electrophoresis was carried out in a BRL model S2 sequencing unit at 75W in 1x TBE. The gel was fixed in 10% methanol (v/v), 10% acetic acid (v/v), dried and autoradiographed overnight.

2.2.12 Autoradiography

Polyacrylamide gels with radiolabelled samples were dried in a slab gel dryer (Hoefer Scientific Instruments) for 1 h at 80°C under vacuum and exposed to Cronex MRF31 X-ray film for appropriate lengths of time. In the case of ^{32}P-labelled samples, intensifying screens were used and exposure was carried out at -70°C.

Table 2.1 The nucleotide sequences and target sites of the oligonucleotide primers used for sequencing of AHSV-3 genome segment 2.

Primer	Sequence	Target
M13/pUC forward	d(GTAAAACGACGGCCAGT)	M13/pUC multiple cloning site
M13/pUC reverse	d(CAGGAAACAGCTATGAC)	M13/pUC multiple cloning site
pBR322-PstI (+)	d(GCTAGAGTAAGTAGTT)	pBR322 (3556-3571)
pBR322-PstI (-)	d(AACGACGAGCGTGAC)	pBR3222 (3638-3624)
SP4	d(AGTAAGCATAAAGCGGA)	AHSV-3.2 (1175-1181)
SP7	d(GCCATGCTCACTCAACTC)	AHSV-3.2 (2337-2320)
SP8	d(CATATATTGACTCCGG)	AHSV-3.2 (1849-1834)

2.2.13 Cloning of full length AHSV-3 genome segment 2 cDNA

Polymerase chain reaction

cDNA synthesized from a pool of large dsRNA genome segments and fractionated by alkaline sucrose gradient centrifugation to yield predominantly large species of cDNA was used as template for the amplification of AHSV-3 segment 2 by the polymerase chain reaction (PCR).
Two oligonucleotide primers, PP1 and PP2, with sequences of

5' CACAGATCTGTTTAATTCACCATGGCTTCG 3' and

5' GAGAGATCTGTAAGTTGATTCACTTGGAGC 3' respectively,

complementary to the 5' terminal sequences of the minus and plus strands of AHSV-3 segment 2 respectively and extended at the 5' ends to include BglII sites (underlined), were synthesized. cDNA synthesized from approximately 50ng large dsRNA segments, 100pmol of each of primers PP1 and PP2, 0.2mM of each dNTP, 10µl 10x Taq polymerase buffer (0.5M KCl, 0.1M Tris-HCl pH9.0, 0.1% gelatin (m/v), 1% Triton X-100) and 6µl 25mM $MgCl_2$ were assembled in a 99.5µl reaction mix and overlayed with 100µl liquid paraffin. Following denaturation of the template at 95°C for 5min, 0.5µl Taq polymerase (Promega, 5 units/µl) was added prior to primer annealing at 49°C for 45sec and elongation at 72°C for 4min. This was followed by a further 30 cycles of denaturation (95°C for 2min), primer annealing (5 cycles at 49°C and 25 cycles at 54°C for 45sec) and elongation (29 cycles for 4 min and 1 cycle for 10 min at 72°C) as detailed in Table 2.2. The PCR products were analysed electrophoretically on a 0.8% agarose gel with DNA molecular weight markers.

Cloning and characterization of the PCR product.

The DNA band corresponding in size to AHSV-3 segment 2 was excised, glassmilk purified and cloned into pBR322 by homopolymeric dG/dC tailing (2.2.3). Plasmids from a number of colonies were isolated and characterized by RE digestion. The insert termini of a selected recombinant were sequenced to confirm the full length AHSV-3 segment 2 clone status.

Table 2.2 Temperature cycling for PCR amplification of genome segment 2 of AHSV-3.

Step	Denaturation		Annealing		Primer extension		No. of cycles
	temp. (°C)	duration (min)	temp. (°C)	duration (s)	temp. (°C)	duration (min)	
1	95	5	49	45	72	4	1
2	95	2	49	45	72	4	5
3	95	2	54	45	72	4	24
4	95	2	54	45	72	10	1

2.3 RESULTS

2.3.1 Purification of viral dsRNA

AHSV-3 was chosen as a candidate for the initial investigation towards the use of VP2 as a subunit vaccine as previous studies of AHSV in South Africa had concentrated on AHSV-3, and to avoid duplication of research of international investigators.

AHSV-3 dsRNA was isolated from infected BHK-21 cells by the SDS-phenol method and purified by MAK column chromatography for the elimination of DNA. In order to obtain dsRNA pools enriched for large genome segments and purified from contaminating tRNA, the dsRNA was fractionated by sucrose gradient centrifugation. The fractions were analysed electrophoretically on agarose gels (Fig 2.1) and combined into three pools containing predominantly large, medium or small genome segments, as indicated. The pool of large genome segments was used in obtaining the AHSV-3 segment 2-specific clone, D2.

2.3.2 cDNA synthesis, analysis and cloning

Three clones, A7, A85 and B9, containing AHSV-3 segment 2-specific cDNA fragments of 0.9, 1.2 and 1.4 kb respectively, were obtained from the OVI. As will be discussed later, however, although both the 5' and 3' termini were found to be included in the cloned fragments, the complete genome segment 2 sequence was not represented. The cloning of additional cDNA fragments was therefore required.

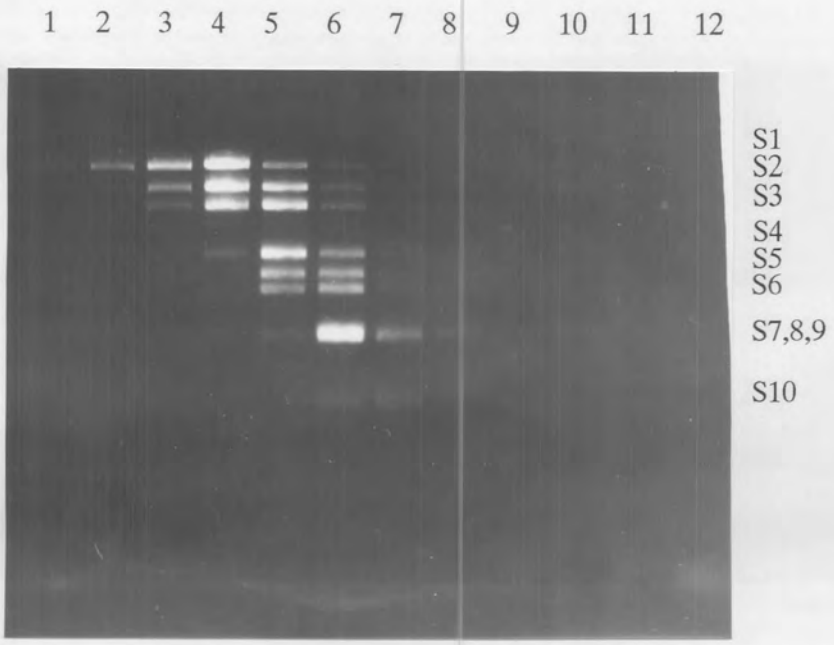

Figure 2.1 An agarose gel electrophoretic analysis of AHSV-3 dsRNA fractionated and purified by sucrose gradient centrifugation. Fractions were combined into pools enriched for large (fractions 1-4), medium (fraction 5) and small (fractions 6-8) genome segments; tRNA is visible eluting from fraction 11. The dsRNA segments (S1-10) are indicated.

Based on literature reports of the strategies employed for the cloning of segmented dsRNA genomes, including the three AHSV-3 segment 2-specific clones obtained from the OVI (Purdy *et al.* 1984; Huismans & Cloete 1987; Bremer *et al.* 1990), the viral dsRNA was originally denatured with MMOH prior to the addition of poly A tails using poly A polymerase. In this study, however, reverse transcription of purified polyadenylated RNA in an oligo dT-primed reverse transcriptase reaction yielded ill-defined cDNA in alkaline agarose gel electrophoretic analyses (results not shown). Although cDNA of this pedigree was successfully utilized for PCR amplification of AHSV-3 segment 2, a joint investigative analysis of polyadenylation of viral dsRNA without prior denaturation, based on a report by Nuss & Dall (1990) of the efficient polyadenylation of nondenatured wound tumor virus (WTV) dsRNA, was undertaken in the Department of Genetics of the University of Pretoria. In this instance, utilizing a pool of dsRNA enriched for medium to small genome segments, reverse transcription yielded distinct oligo dT-primed cDNA species on alkaline agarose gels in a pattern recognisable as corresponding to electrophoretically-separated AHSV-3 genome segments (Fig 2.2).

Hydrolysis of the RNA template by resuspension of the cDNA in a 0.3N NaOH buffer was followed by size fractionation of the cDNA by alkaline sucrose gradient centrifugation and analysis of the fractions by Cerenkov counting (Fig 2.3A). Samples of the cDNA fractions in the ascending portion of the peak were analysed by alkaline agarose gel electrophoresis (Fig 2.3B) and pooled as indicated in Fig 2.3A. Alternatively, the cDNA was size fractionated by electrophoretic separation on alkaline agarose gels, detected by autoradiography and isolated in gel slices as pools of segments 1-3, 4-6 and 7-10 (results not shown due to indistinctness of the autoradiograph). Only pools of large cDNA species were used for further cloning.

Approximately 70-80% of the cDNA was recovered from the alkaline sucrose gradient pools or the alkaline agarose gel slices by Geneclean™ extraction. Following the addition of dC tails to the 3' ends of the cDNA in a C-tailing reaction, the cDNA was annealed to dG-tailed PstI-cut pBR322. Although this cloning procedure yields recombinants with homopolymer dG/dC tracts flanking the inserts, it represents an efficient technique to clone small amounts of cDNA. The annealed DNA was transformed into competent HB101 cells and plated out on LB agar plates containing tet and incubated overnight at 37°C; however, it was found that a further 24 h incubation yielded significant numbers of additional colonies. Plasmids of transformants were isolated and analysed by comparison with intact pBR322 controls in agarose gel electrophoresis. The larger recombinant plasmids were further characterized by genome segment assignment.

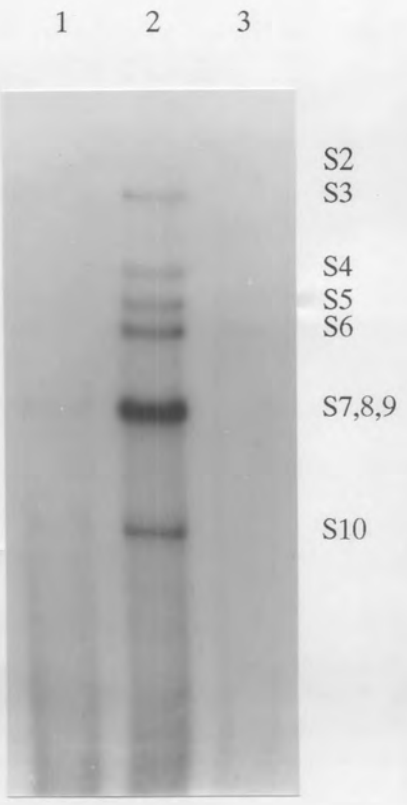

Figure 2.2 An autoradiograph of an alkaline agarose gel electrophoretic analysis of oligo dT-primed AHSV-3 specific cDNA. The template dsRNA in each case was either denatured (lane 1) or left undenatured (lane 2) for polyadenylation. Lane 3 represents an unprimed cDNA synthesis reaction as a negative control. The cDNA segments (S2-10) are indicated.

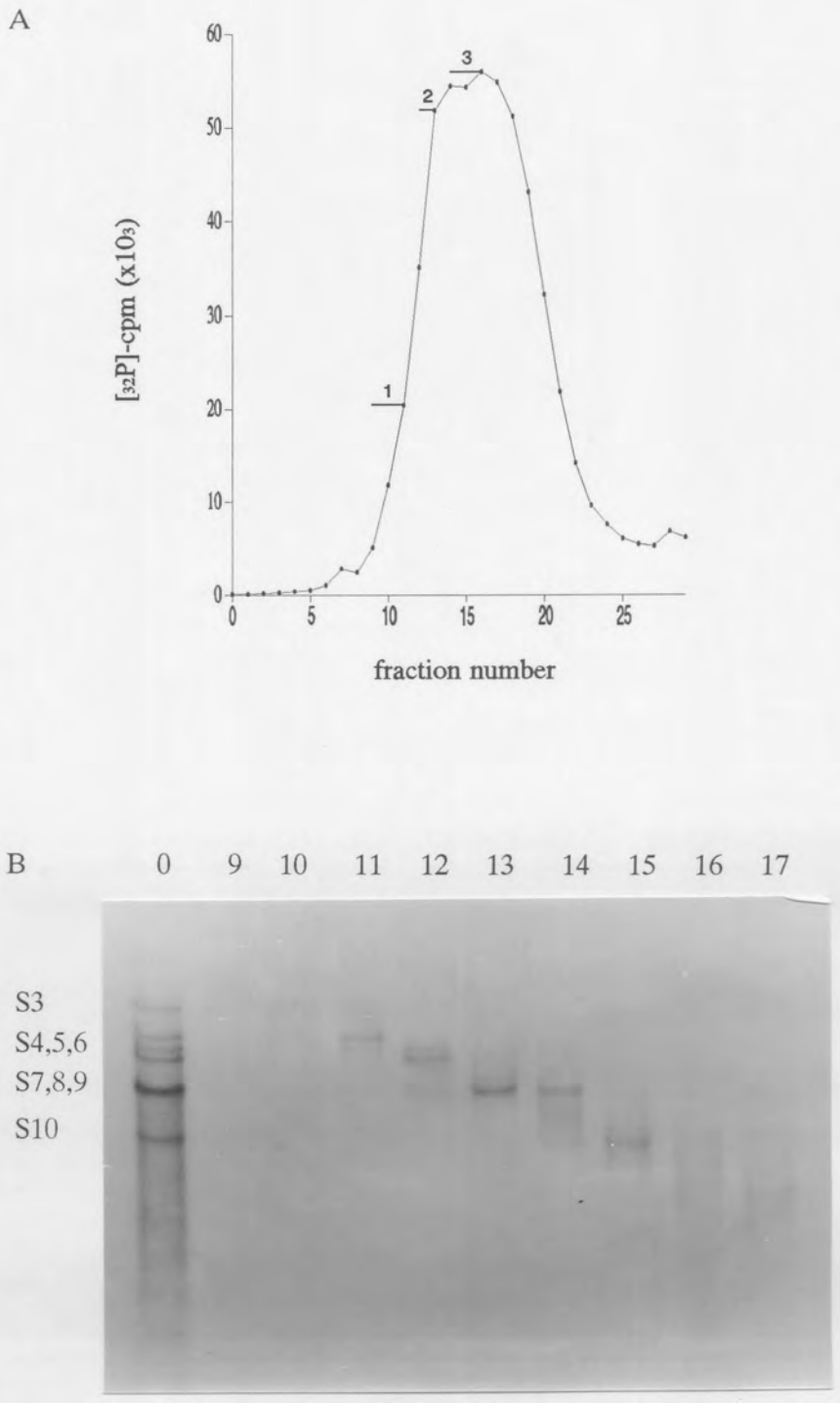

Figure 2.3 Size separation of AHSV-3 specific cDNA by alkaline sucrose gradient centrifugation. Fractions were analysed by Cerenkov counting and pooled as indicated (A). Samples of the fractions in the ascending portion of the peak (fractions 9-17) were analysed by alkaline agarose gel electrophoresis and autoradiography (B); lane 0 represents an unfractionated cDNA pool. The cDNA segments (S3-10) are indicated.

2.3.3 Characterization of clones

AHSV-3 dsRNA was separated by preparative 6% PAGE and blueprinted prior to denaturation and blotting onto nylon membrane. Selected recombinant plasmids were labelled by nick translation and hybridized to these Northern blots. Following autoradiography, the segment representation of the clones was determined by alignment of positive signals on the autoradiograph with the blueprint of genome segments on the membrane (Fig 2.4). The assignment of the clones obtained from the OVI, namely A85, B9 and A7, as AHSV-3 segment 2-specific, was also confirmed by this technique.

The AHSV-3 segment 2-specific clones used in this investigation, along with the largest clones of each of the AHSV-3 and AHSV-9 genome segments 1 to 5 obtained by various researchers in the Department of Genetics of the University of Pretoria, are summarised in Table 2.3. The size of the inserts were estimated by excision with PstI, since the cloning procedure restores the PstI sites at both ends of the cloned insert, and comparison of their mobilities with those of HindIII-restricted λ DNA and HaeIII-restricted ϕX174 DNA molecular weight markers of known size in agarose gel electrophoresis. Further screening by hybridization of A85 and D2 inserts, Geneclean™-purified from agarose and labelled by nick translation, to dotblots of recombinant plasmid DNA yielded no additional segment 2-specific clones.

2.3.4 Characterization of AHSV-3 genome segment 2

Four AHSV-3 segment 2-specific clones designated A85, D2, A7 and B9 were identified. In order to construct subclones for sequencing purposes, limited restriction maps of the clones were prepared by individual or combined digestion with AccI, BamHI, BglI, BglII, ClaI, HindIII, KpnI, PstI, PvuI, SacI and XbaI. Based on these maps and in conjunction with a restriction map of pBR322 (Sambrook *et al.* 1989), subclones of A85 and B9 were constructed in pUC13 and M13mp19 as indicated in Table 2.4. The latter vectors are useful for sequencing as both contain a multiple cloning site comprised of a number of unique restriction sites in close proximity to each other, facilitating subcloning of fragments, and bordered by recognition sites for forward and reverse sequencing primers. RE fragments were Geneclean™-purified from agarose gels and ligated to similarly purified RE-linearized dephosphorylated vector DNA. Following transformation into competent *E. coli* cells, recombinant plasmids were identified by RE digestion and purified by CsCl density gradient centrifugation.

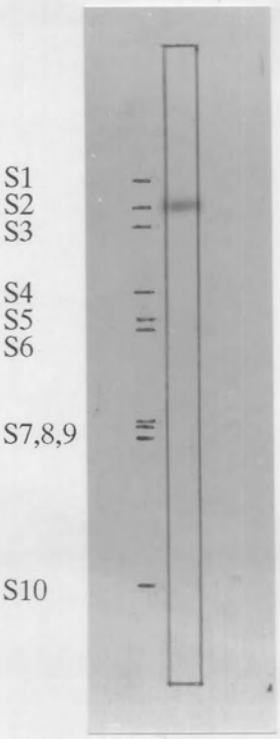

Figure 2.4 A northern blot hybridization analysis of recombinant D2 to electrophoretically separated AHSV-3 dsRNA. The positions of the ten genome segments on the membrane are indicated.

Table 2.3 Summary of the sizes and origins of some of the AHSV-specific clones obtained by various researchers in the Department of Genetics of the University of Pretoria. The AHSV-3 genome segment 2-specific clones used in this investigation are indicated, along with the largest clones of AHSV-3 and AHSV-9 genome segments 1 to 5 obtained.

Clone	Genome segment	AHSV serotype	size (kb)	nature of source RNA[1]	cDNA source[2]
I31	1	9	3.8*	undenatured	gradient
A7	2	3	0.9	denatured	gradient
A85	2	3	1.2	denatured	gradient
B9	2	3	1.4	denatured	gradient
D2	2	3	2.1	undenatured	gel
D9	3	3	2.8*	undenatured	gel
I74	3	9	2.8*	undenatured	gradient
G7	4	3	2.0*	undenatured	gradient
I71	4	9	2.0*	undenatured	gradient
G15	5	3	1.5*	undenatured	gradient
I76	5	9	1.5*	undenatured	gradient

[1] dsRNA denatured or left undenatured for polyadenylation.

[2] cDNA fractionated by alkaline sucrose gradient centrifugation or by alkaline agarose gel electrophoresis.

* complete or almost complete genome segment.

Table 2.4 Summary of the sizes and origins of the clones and subclones used for sequencing of AHSV-3 segment 2.

Clone	Vector	Restriction fragment	Fragment size (bp)	AHSV-3.2 represented
A85	pBR322	PstI*/PstI*	1201	1-1201
A85.B	pUC13	BamHI*/BamHI	1154	1-29
A85.BP	pUC13	BamHI/PstI*	1171	30-1201
A85.C1	M13mp19	ClaI*/ClaI	1305	1-531
A85.C2	M13mp19	ClaI/ClaI	510	532-1042
pAHSV3.2**	pBR322	PstI*/PstI*	3221	1-3221
D2	pBR322	PstI*/PstI*	2006	1215-3221
B9	pBR322	PstI*/PstI*	1438	1783-3221
A7	pBR322	PstI*/PstI*	924	2279-3203
B9.XP	M13mp19	PstI*/XbaI	917	1783-2700
B9.C	M13mp19	ClaI*/ClaI	2019	1783-3028

* pBR322-specific restriction site

** clone derived from PCR amplification

Sequencing was carried out with the appropriate pBR322 (+) or (-) primers, M13/pUC forward or reverse primers or with synthetic oligonucleotide primers based on previously determined sequences (Table 2.1 in Materials and Methods). Occassionally, notably in the sequencing of the termini of A85 and D2 with the pBR322 (-) primer, AHSV-3 segment 2-specific sequences could not be reliably determined due to sequence compressions caused by pausing of the polymerase enzyme in regions of exceptional secondary structure, such as are brought about by homopolymeric dG/dC tails. Such sequences were verified by sequencing with synthetic primers and/or sequencing of subclones of internal restriction fragments.

The strategy employed for the sequencing of the entire AHSV-3 segment 2 genome segment is shown in Fig 2.5. The regions of the segment 2 gene represented in the different clones, and the portion of each clone which was sequenced, are indicated.

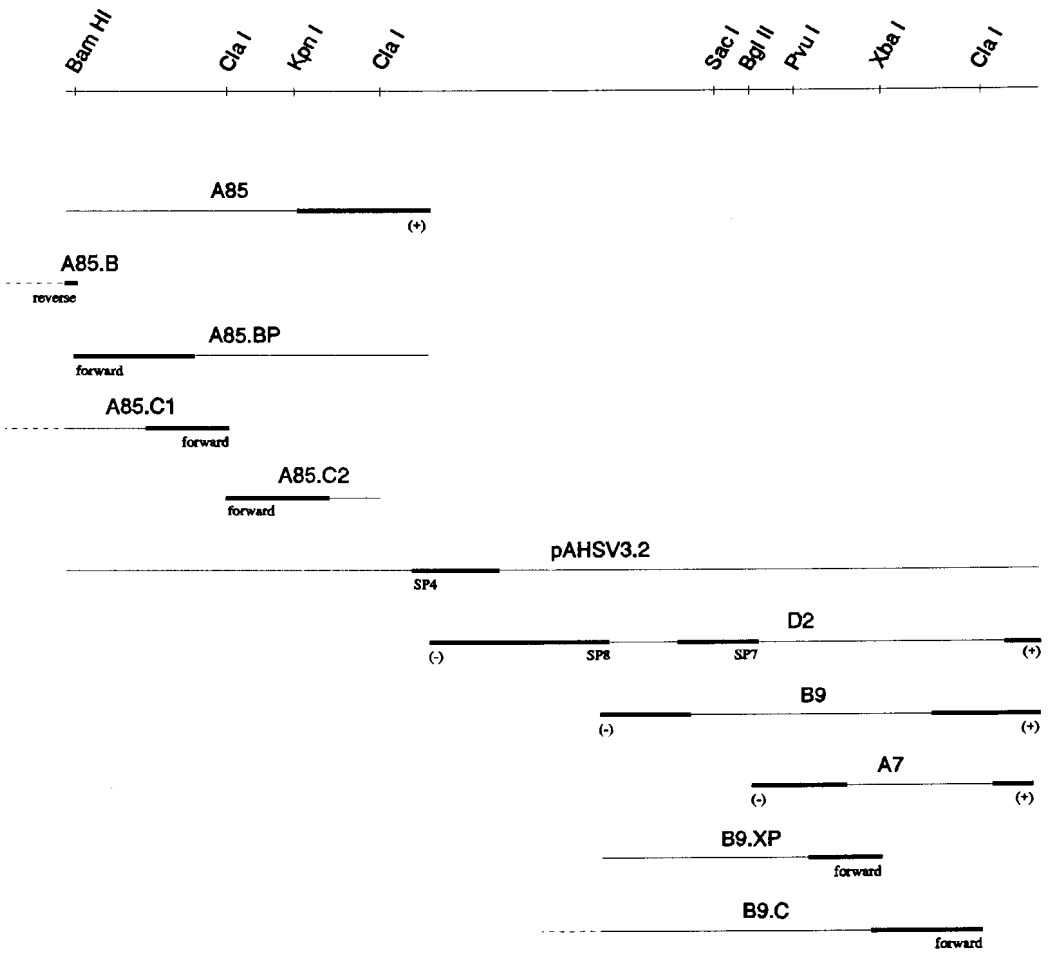

Figure 2.5 A schematic representation of a restriction map of AHSV-3 genome segment 2 and the relative positions of the clones and subclones used as templates for the sequencing of the gene. The solid lines represent the different clones and the bold sections indicate portions of the clone sequenced with the specified primer. Vector-specific sequences included during subcloning are indicated by dotted lines.

By merging the sequences, the complete AHSV-3 genome segment 2 nucleotide sequence, as given in Fig 2.6 together with the predicted amino acid sequence of the VP2 protein, was deduced to be 3221 bp long with a calculated base composition of 31.20% A, 26.05% G, 27.63% T and 15.12% C.

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GTTTAATTCACCATGGCTTCGGAATTCGGGATCCTATTGACAAATCAAATATATGATCAAACATATGAGAAAGAGATGTGTGATGTAATTATTACAGCGGAGAATGCAGTTAGAAGAGTT  120
                M  A  S  E  F  G  I  L  L  T  N  Q  I  Y  D  Q  T  Y  E  K  E  M  C  D  V  I  I  T  A  E  N  A  V  R  R  V

GAGGTTGCGGGAGTACATGGTTATGAGTGGGGTGCGACGAATCATAGGCTTGGGTTGTGTGAGGTAGAGAACACGAAGTCGATTGGGAGAATGATTTATGAACAGATTCGATGCGAAGGT  240
  E  V  A  G  V  H  G  Y  E  W  G  A  T  N  H  R  L  G  L  C  E  V  E  N  T  K  S  I  G  R  M  I  Y  E  Q  I  R  C  E  G

GCGTACCCAATTTTTCCGCACTATATAACGGATACTCTAAAATATGGGAAATCAATTGATAGAAATGACAACCAAATAAGGGTGGATAGGGATGATGAACGCGTCCGCAAAATAAAAATT  360
  A  Y  P  I  F  P  H  Y  I  T  D  T  L  K  Y  G  K  S  I  D  R  N  D  N  Q  I  R  V  D  R  D  D  E  R  V  R  K  I  K  I

CAGCCGTATTTTGGAGAAATGTACTTTTCACCAGAAAATTATATAACAGTTTTTTGTAAAAGGCAGGCAATCAGTGGCCAAATTGAGGTTTCTCGTTCAATTATAGGTCGGCGGATGAAG  480
  Q  P  Y  F  G  E  M  Y  F  S  P  E  N  Y  I  T  V  F  C  K  R  Q  A  I  S  G  Q  I  E  V  S  R  S  I  I  G  R  R  M  K

TATGAAGAGAGTGCTGAACAGACGAAGGGAACGATAAACGCAAATAAATATCGATTGTTGGAGAAGTGGCGCGATCTGGCCTATGAACAAATTGAAATAGAACGTGATAATGAAAGATGT  600
  Y  E  E  S  A  E  Q  T  K  G  T  I  N  A  N  K  Y  R  L  L  E  K  W  R  D  L  A  Y  E  Q  I  E  I  E  R  D  N  E  R  C

TTGACGCATAACACCGACCCGATTTATCAATTGATAAAAAAGATGAGGTATGGAATGATGTATCCAGTGCACTACATGCTAAACGATAGATATAAAGTTGTGCAAGAGCGCGCAGATATG  720
  L  T  H  N  T  D  P  I  Y  Q  L  I  K  K  M  R  Y  G  M  M  Y  P  V  H  Y  M  L  N  D  R  Y  K  V  V  Q  E  R  A  D  M

GGGGTTGAAAAGTGGCTTCTTCAAAAAATCGGAAGGGGTACCCAAAGGCGGAAGGCTGACGATGGCGATAATGATGCGCTCTTACAGCTAGAAAGAATGATGAGCAGTGAAGAGCTGGAA  840
  G  V  E  K  W  L  L  Q  K  I  G  R  G  T  Q  R  R  K  A  D  D  G  D  N  D  A  L  L  Q  L  E  R  M  M  S  S  E  E  L  E

AGATCCGTTATTGAGAGCGTCATAAGATTCGGTTCTTTATATAACGCGCACGCGGGCAAAAAGACAGGCGACATTCCATTAGAAGTCTTGATTAAATATTGCGATTCGCTTACTACATTC  960
  R  S  V  I  E  S  V  I  R  F  G  S  L  Y  N  A  H  A  G  K  K  T  G  D  I  P  L  E  V  L  I  K  Y  C  D  S  L  T  T  F

GTGCATAAAAAGAACAGAGAGGGAGGAGATAATCAGACCGCTCGCGACGAGATTAGGAGGGCAGTGGTAAAGAATATTCCATCGATGAAACAGGAGAATCAGATGAAAGTCACTCCTAAT 1080
  V  H  K  K  N  R  E  G  G  D  N  Q  T  A  R  D  E  I  R  R  A  V  V  K  N  I  P  S  M  K  Q  E  N  Q  M  K  V  T  P  N

ATCAGGAATTTTCTGTTTTTCGCATATTTAAACGGTTTTAAAAGGAATAATGGCGTTGATATCGACCCAAATAATGGTACGTGGAGTAAGCATAAAGCGGAAGTTAAGAAGTTTTTGAAC 1200
  I  R  N  F  L  F  F  A  Y  L  N  G  F  K  R  N  N  G  V  D  I  D  P  N  N  G  T  W  S  K  H  K  A  E  V  K  K  F  L  N

GAGGAACAAAAGAAGAATGAAAATAAACCATTAAAGGTGTTGATTGATGGCGCGTACATATCAACCGATGCTGAGTACGGAACCGTAGCGCATTGGGTGGATTGGGTCGTTGATATCGTA 1320
  E  E  Q  K  K  N  E  N  K  P  L  K  V  L  I  D  G  A  Y  I  S  T  D  A  E  Y  G  T  V  A  H  W  V  D  W  V  V  D  I  V

ATGATGACTCAAGTTAGCCGCATGATAAAAGAATATAATTTTATTAGATTAAAAAAGGATCAGCTAATCAGCGGTATGAATAAGTTGGAGGATGGGGTAAAATGCTACGCGTATTGTTTG 1440
  M  M  T  Q  V  S  R  M  I  K  E  Y  N  F  I  R  L  K  K  D  Q  L  I  S  G  M  N  K  L  E  D  G  V  K  C  Y  A  Y  C  L

ATCCTCGCACTGTACGATTTTCATGGGCGTGACGTAGACGGCTTTGCGCAGGGGACGAGAACCGCAGCTATTGTTGAGACCGTCGCGCGAATGTTTCCTGATTTTCGCTCTGAGGTTTCG 1560
  I  L  A  L  Y  D  F  H  G  R  D  V  D  G  F  A  Q  G  T  R  T  A  A  I  V  E  T  V  A  R  M  F  P  D  F  R  S  E  V  S

GAAAAATTCGGTATTGATTTAGCGGTGTCAGAGGAATCAGATGAACTATTCGTAAAGAAGACGATGGTCTCGAGTTTCTCTGACTCTGGGGAGATGGGTTACAAATTCATATTTGGATGG 1680
  E  K  F  G  I  D  L  A  V  S  E  E  S  D  E  L  F  V  K  K  T  M  V  S  S  F  S  D  S  G  E  M  G  Y  K  F  I  F  G  W

AGGAAAACTGATTTCAAGGTTGAGACTGATTATGGAGAGATAGTTTCTGATGAAGTCCATCGGTTATATCAAGCAATTTTGGATGGCAAGGAATGGAGTAAAGAGGTTGATGACCCTGAA 1800
  R  K  T  D  F  K  V  E  T  D  Y  G  E  I  V  S  D  E  V  H  R  L  Y  Q  A  I  L  D  G  K  E  W  S  K  E  V  D  D  P  E

AAATACTTCGTTGATGATTTATATAATAGATGCCCGGAGTCAATATATGTCAGGAACGGAGTTGATCCTAATAATAAGATAATGATTAAGAAGCGAGGTTTAGTTGGGGAGAGCCAGCGC 1920
  K  Y  F  V  D  D  L  Y  N  R  C  P  E  S  I  Y  V  R  N  G  V  D  P  N  N  K  I  M  I  K  K  R  G  L  V  G  E  S  Q  R

ATTTTTCTGCGAGATTTGTCTCATATTGGTATGAATTTCAAAAAGTTACTATTGAGGCTGAGTTCGAAGCGATTGCACGCACGTGGCGAACATATACAGTATCATGAGATAGATGTTGAA 2040
  I  F  L  R  D  L  S  H  I  G  M  N  F  K  K  L  L  L  R  L  S  S  K  R  L  H  A  R  G  E  H  I  Q  Y  H  E  I  D  V  E

GATTTCAAACCTTGCGCGATTGCGGAACTTGGATTACATTGCTCAACCTATATTTATCAAGATTTGCTCGTTGGCGCGAACAGAGGTGAATATGTGAAGGATGCGAAGGAGCTCGTCTGG 2160
  D  F  K  P  C  A  I  A  E  L  G  L  H  C  S  T  Y  I  Y  Q  D  L  L  V  G  A  N  R  G  E  Y  V  K  D  A  K  E  L  V  W

TTCGATATCGCTAACACAAACTTCAACATCACGCGTCCTTTTGATAGATGCTGGCCTTCTTCTTGCGCGGAGGCTGAGTTATCCTTGAGGTTTCATCTGATTACCAAGATCTTCACGAGA 2280
  F  D  I  A  N  T  N  F  N  I  T  R  P  F  D  R  C  W  P  S  S  C  A  E  A  E  L  S  L  R  F  H  L  I  T  K  I  F  T  R

TATCGAGGCGAAAGAACTTCGTTTGTCGATATAATTAATGAGTTGAGTGAGCATGGCTACGTGAAACACAATTTCCCGTCATATAAGCATTATTACCTCTCTGTTATTCAGACGGTTTTT 2400
  Y  R  G  E  R  T  S  F  V  D  I  I  N  E  L  S  E  H  G  Y  V  K  H  N  F  P  S  Y  K  H  Y  Y  L  S  V  I  Q  T  V  F

GAGGATCAAAGAGCGATCGATCCGCTTGACTTTTGTGCAATGATTTCACGGAATGAGACGCGAGAGAGCACGCTCAAGGGATTTAGTATGTTTACTGCAATTGTTAAAAGTGAGAGACTG 2520
  E  D  Q  R  A  I  D  P  L  D  F  C  A  M  I  S  R  N  E  T  R  E  S  T  L  K  G  F  S  M  F  T  A  I  V  K  S  E  R  L

ATAGATACCCTGTTCTTGAATTTCTTGTTATGGATTGTCTTTGAAATGGAGAATGTTGATGTGAGCGCTGCTAATAAGAGACATCCATTATTAATATCGCATGAAAAAGGATTACGTTTA 2640
  I  D  T  L  F  L  N  F  L  L  W  I  V  F  E  M  E  N  V  D  V  S  A  A  N  K  R  H  P  L  L  I  S  H  E  K  G  L  R  L

ATTGGCGTAGATTTGTTTAATGGCGCGCTTTCGATTTCCACGGGGGGGTGGATTCCGTATCTAGAGAGGATATGTTCAGAGGAGAAAGCTCAGAGAAGGTTGAACGCGGATGAACTGAAA 2760
  I  G  V  D  L  F  N  G  A  L  S  I  S  T  G  G  W  I  P  Y  L  E  R  I  C  S  E  E  K  A  Q  R  R  L  N  A  D  E  L  K

ATAAAAAGTTGGTTTTTAACGTATTATATGAATCTTTCGTTAGAAAGGAGAGCGGAGCCGCGTATGAGCTTTAAGTTCGAAGGGTTAACCACTTGGATCGGCTCAAATTGTGGTGGAGTT 2880
  I  K  S  W  F  L  T  Y  Y  M  N  L  S  L  E  R  R  A  E  P  R  M  S  F  K  F  E  G  L  T  T  W  I  G  S  N  C  G  G  V

CGCGATTATGTCGTCCAAGCTCTACCGATGCGGAAACCAAAGCCTGGTTTATTGATGATAATTTATGGAGACGATGGGGACGCGCGTTGGGTAGAGTGGGCAATGAAGAATTTTACAGCG 3000
  R  D  Y  V  V  Q  A  L  P  M  R  K  P  K  P  G  L  L  M  I  I  Y  G  D  D  G  D  A  R  W  V  E  W  A  M  K  N  F  T  A

GTTGATGGATCGTTGGGCTTCATTTATATCGATAGACATAAGCTGGTTAACAAGAGTGATTTCCGAGTCAGAGAAATGAAAATATATAACCGAGGACGTTTAGACCGTCTGATATTGATA 3120
  V  D  G  S  L  G  F  I  Y  I  D  R  H  K  L  V  N  K  S  D  F  R  V  R  E  M  K  I  Y  N  R  G  R  L  D  R  L  I  L  I

TCTAGTGGTCATTATACATTTGGGAATAAGTTTCTAATGTCTAAGCTGCTTGCGAAAACTGAATAAGCGGAGTGACTCCCGCTCCATGTGAATCAACTTAC  3221
  S  S  G  H  Y  T  F  G  N  K  F  L  M  S  K  L  L  A  K  T  E  U
```

Figure 2.6 The nucleotide sequence of cDNA of the minus strand of AHSV-3 genome segment 2 and the predicted amino acid sequence of the translation product.

The longest open reading frame (ORF) identified was defined by an ATG at position 13-15 and a TAA at position 3184-3186, delineating 5' and 3' noncoding regions of 12 bp and 35 bp respectively. The presence of a purine (A) in position -3 and a G in position +4 satisfies the model proposed by Kozak (1988) for initiation of protein synthesis at this ATG.

Based on the cDNA sequence, the AHSV-3 segment 2 gene encodes a protein comprised of 1057 amino acids with an estimated Mr of 123,078 kDa. The indicated VP2 protein contains a large proportion of charged residues (R, H, K, D and E), representing 31% of the total amino acids. Assuming that glutamic and aspartic acids each have charges of -1, arginine and lysine +1 and histidine +0.5 at neutral pH, the AHSV-3 VP2 protein has a calculated net positive charge of 15 at pH7.0.

2.3.5 Cloning of full length AHSV-3 genome segment 2 cDNA

As no single full length cDNA clone of AHSV-3 genome segment 2 was obtained, an alternative strategy for the cloning of the complete VP2 gene suitable for subsequent expression, was devised. AHSV-3 segment 2 cDNA was PCR-amplified with two synthetic oligonucleotide segment 2 termini-specific primers, PP1 and PP2 (Fig 2.7), simultaneously incorporating terminal BglII sites, basically as described by Nel & Huismans (1991).

```
PP1: 5' CACAGATCTGTTTAATTCACCATGGCTTCG 3'
S2 :          5' GTTTAATTCACCATGGCTTCGGAATTCGGG......
                 ........................................
                 .....TAAGCGGAGTGACTCCCGCTCCATGTGAATCAACTTAC 3'
PP2:                                  5' CGAGGTTCACTTAGTTGAATGTCTAGAGAG 3'
                                                 *
```

Figure 2.7 The 5' and 3' terminal sequences of cDNA of the AHSV-3 genome segment 2 minus strand and the corresponding sequences of the oligonucleotide primers, PP1 and PP2, for PCR amplification of segment 2-specific cDNA. The VP2 gene initiation and stop codons are underlined, and the oligonucleotide-specific BglII restriction sites are indicated in bold. The asterisk marks a sequence mismatch in PP2.

A single nucleotide error in the sequence of the 3' terminal-specific primer, PP2, in terms of its complementarity to segment 2, was ignored as the relevant sequence was located in the non-coding region of the gene. Besides the ubiquitous primer dimers, a single 3.2 kb

band representing AHSV-3 segment 2 was obtained in an electrophoretic analysis of the PCR product (Fig 2.8). The DNA fragment was isolated and cloned into pBR322 by homopolymeric dG/dC tailing, as the presence of an internal BglII restriction site complicated digestion of the terminal BglII sites incorporated in the primers. A clone containing the complete AHSV-3 segment 2, based on BglII restriction analysis, was obtained and designated pAHSV3.2. Limited sequencing of the termini was carried out to verify the integrity of the clone. At least two point mutations were identified involving substitution of T at position 38 with C and A at position 3080 with G. In addition, in the latter region, no band corresponding to a DNA fragment ddNTP-terminated at position 3081 was visible on the autoradiograph (Fig 2.9), suggesting an inability of the complementary ddNTP to bind to the template at this position. The reason for this is unknown. These mutations can be ascribed to the comparatively high misincorporation rate of Taq DNA poymerase, which has been estimated at $2x10^{-4}$ per nucleotide per cycle, corresponding to an overall error frequemcy of 0.25% after 30 cycles (Saikki *et al.* 1988). The mutations give rise to Leu to Ser and Lys to Ser or Arg substitutions at amino acids 9 and 1023 respectively in the encoded VP2. It was nonetheless decided to proceed with expression of the cloned gene as described in Chapter 3.

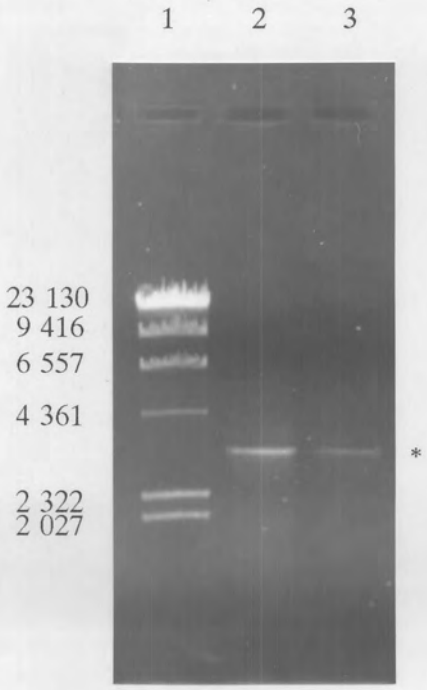

Figure 2.8 An agarose gel electrophoretic analysis of PCR amplifications of AHSV-3 genome segment 2-specific cDNA. Lane 1 represents a DNA molecular weight marker, with fragment sizes as indicated in bp. Lanes 2 and 3 represent 10µl of 100µl PCR reaction mixes containing 1.5mM and 2.5mM $MgCl_2$ respectively. The asterisk indicates the amplified AHSV-3 segment 2 cDNA.

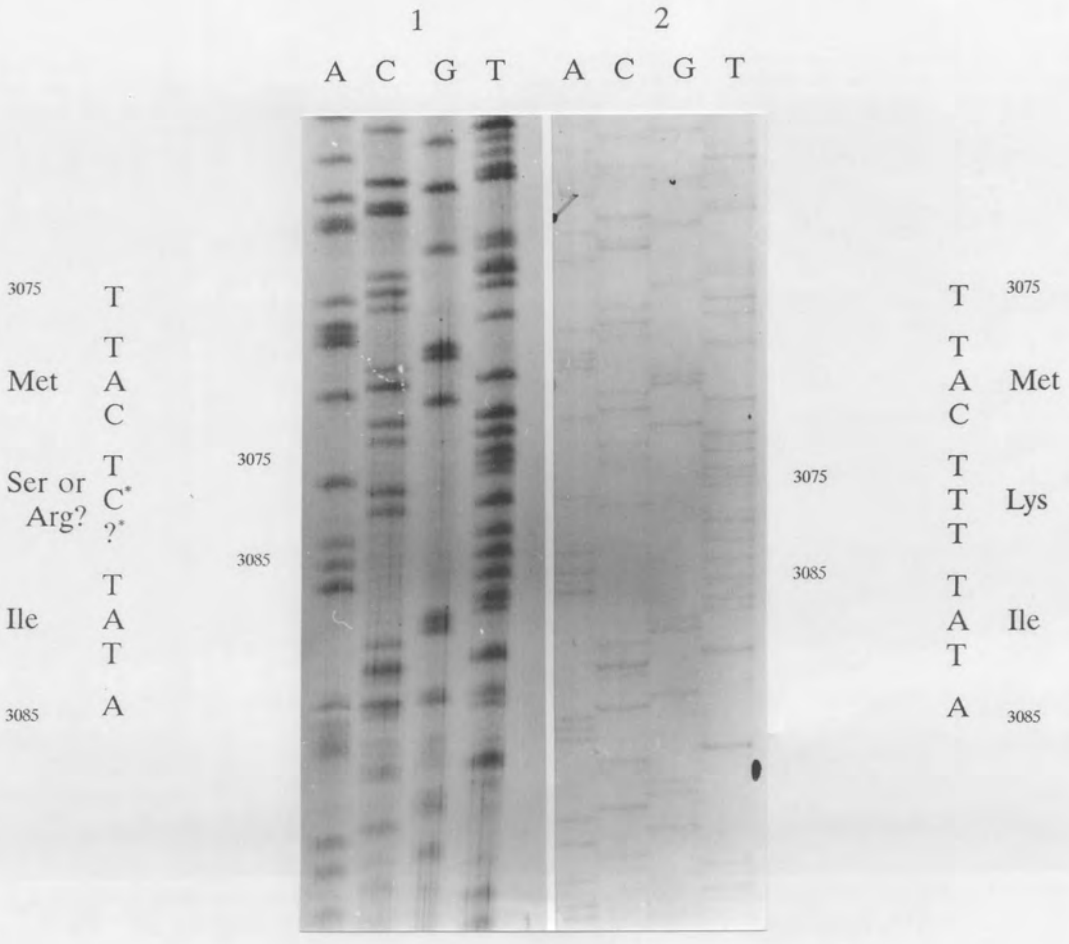

Figure 2.9 Autoradiographs of portions of the PAGE nucleotide sequence analyses of pAHSV3.2 (lane 1) and B9 (lane 2). The relevant sequences representing bases 3072-3089 of AHSV-3 genome segment 2, and the translated amino acids (residues 1022 to 1024), are indicated. The bases labelled with asterisks represent misincorporations in the PCR product.

2.4 DISCUSSION

In this chapter, the cloning and characterization through nucleotide sequence determination of the segment 2 gene of AHSV-3 has been described, and the corresponding amino acid sequence of the encoded VP2 protein deduced.

The cloning of the dsRNA genome segments as cDNA of a number of different members of the Reoviridae family has been described, including the complete genomes of reovirus serotype 3 (Cashdollar *et al.* 1984), BTV-10 (Fukusho *et al.* 1989) and rotavirus SA11 (Mitchell & Both 1990). Besides the expression of the encoded protein, cloning of the dsRNA genome segments greatly facilitates investigation into the molecular biology of the gene and the gene product. Characterization of the cloned gene through nucleotide sequencing allows derivation of the amino acid sequence of the encoded protein, enables detailed comparisons of cognate genes and proteins and provides insight into the possible functioning of the protein.

The AHSV-3 VP2 gene was cloned using a modification of the combined strategies described by Huismans & Cloete (1987, originally from Cashdollar *et al.* 1984) and Kowalik *et al.* (1990). The former strategy involves polyadenylation of MMOH-denatured dsRNA and oligo dT-primed cDNA synthesis followed by size fractionation and cloning into pBR322 by homopolymeric dG/dC-tailing. A number of partial AHSV-3 segment 2-specific cDNA clones prepared by this method were obtained from the OVI. Kowalik *et al.* (1990) described the cloning of full length DNA copies of dsRNA genes by selective amplification using PCR. In this study, AHSV-3 segment 2 termini-specific synthetic oligonucleotides, based on sequences obtained from the partial cDNA clones, were used for segment 2-specific amplification of cDNA prior to cloning of the full length segment 2 by homopolymeric dG/dC-tailing. Simultaneously, a BglII site was incorporated immediately adjacent to both the 5' and 3' termini, allowing cloning of the full length gene without the flanking homopolymeric dG/dC sequences. This strategy, previously described by Dennis (1990) and Nel & Huismans (1991) has been successfully used in our laboratory to modify and clone copies of the dsRNA genes encoding VP3, VP4, VP5 and VP7 of different AHSV strains (unpublished results, Department of Genetics, University of Pretoria) and NS2 (Uitenweerde *et al.* submitted for publication) and NS3 (Van Staden *et al.* in press) of AHSV-3.

We have subsequently modified the method of Huismans & Cloete (1987) for cDNA synthesis by the polyadenylation of non-denatured dsRNA, resulting in an apparently proportionally increased yield of full length cDNA species following reverse transcription, as opposed to incomplete cDNA fragments. It is proposed that denaturation of the dsRNA exposes 3' ends of single-stranded nicks in the RNA to polyadenylation. These non-terminal polyadenylated sites could then serve as priming sites for oligo dT, yielding incomplete cDNA

fragments. Although no full length segment 2 clones were obtained, a number of apparently full length clones of segments 1 to 5 were obtained from cDNA reverse transcribed from RNA which had been polyadenylated without prior denaturation, illustrating the advantages of the latter technique.

The entire AHSV-3 segment 2 nucleotide sequence was determined from the partial cDNA clones, excepting a short region comprising 14 bp that was obtained from the PCR product. This represents only the second complete AHSV genome segment 2 sequence obtained.

The AHSV-3 segment 2 gene is 3221 bp long with a coding region of 3174 bp and 5' and 3' noncoding regions of 12 and 35 bp respectively. For further analysis of the sequence by comparative studies, cognate orbiviral gene sequences were retrieved from the Genbank computer database under the following accession numbers:

AHSV-4 segment 2	.....	M94680
EHDV-1 segment 2	.....	D01140
BTV-1 segment 2	.....	X55800
BTV-2 segment 2	.....	M21946
BTV-3 segment 2	.....	X55801
BTV-10 segment 2	.....	M11787
BTV-11 segment 2	.....	M17437
BTV-13 segment 2	.....	D00153
BTV-17 segment 2	.....	M17438

The members of the Reoviridae family, including the *Orbivirus* genus, have short regions of terminal sequences which are conserved within a given genus (Antczak *et al.* 1982; Gaillard *et al.* 1982; Mertens & Sanger 1985; Eiden *et al.* 1992). Based on comparisons of all the published sequences of AHSV genes, Mizukoshi *et al.* (1993) proposed AHSV consensus terminal sequences of 5' $GUU^{A}_{U}A^{A}_{U}$ and $^{A}_{C}C^{A}_{U}UAC$ 3', which are similar, but not identical, to that reported for BTV and EHDV RNA. The terminal sequences present in all known segments of BTV and EHDV are 5' GUUAAA and ACUUAC 3' (Rao *et al.* 1983; Mertens & Sanger 1985; Nel *et al.* 1990). The 5' and 3' terminal hexanucleotides of the AHSV-3 genome segment 2, as determined in this study, 5' GUUUAA and ACUUAC 3', support the AHSV consensus. The deviation from other orbiviral terminal hexanucleotide consenses and the less conserved character seen in AHSV terminal sequences were also reported in Tilligerry virus, a member of the Eubenangee orbivirus serogroup (5' $GU^{AA}_{UU}A^{A}_{U}$ and $AC^{A}_{U}UAC$ 3'; Mertens & Sangar 1985) and Broadhaven virus (5' GU^{AAAA}_{UGGC} and $^{A}_{G}GAUAC$ 3'; Moss *et al.* 1990; 1992a, b).

Alignments of the 5' and 3' noncoding regions of the segment 2 mRNA of selected

orbiviruses are shown in Fig 2.10.

```
AHSV-3      5' GUUUAAU    U     CACCAUG......
AHSV-4      5' GUUAAAU    U     CACUAUG......
EHDV-1      5' GUUAAAU    UGUGUCAGGAUG......
BTV-10      5' GUUAAAAGAGUGUUCUACCAUG......
                *** **     *      *  ***

AHSV-3      ......UAAGCGGAGUGACUCCCGCU  CCAUGUGAAUCAACUUAC 3'
AHSV-4      ......UA GCAACGUGACUGUUGCU  CCAUGUGAAUACACAUAC 3'
EHDV-1      ......U GAUCCGGUGGAACGGAUCACCUGACACAACACGCUUAC 3'
BTV-10      ......UAGGUCCUGUGACAUGGACCGG UAGCCUCUUACACUUAC 3'
                    *       ***                        * ***
```

Figure 2.10 Best fit alignments of the 5' and 3' noncoding regions of the segment 2 mRNA of AHSV-3, AHSV-4, EHDV-1 and BTV-10. The * symbols denote conserved residues.

The sequences adjacent to the 5' and 3' termini of AHSV-3 segment 2 were identified as inverted repeats (Fig 2.11). The repeat sequences comprised 14 bp with a homology of 85.7%. Short segment-specific inverted repeats were first identified in the segmented ssRNA genome of influenza virus (Hsu *et al.* 1987; Stoeckle *et al.* 1987). Similar inverted repeats can been shown in published sequences of all BTV segments (Roy 1989), and have also been reported for EHDV (Nel *et al.* 1990) and other dsRNA viruses (Anzola *et al.* 1987; Stoeckle *et al.* 1987; Moss *et al.* 1992a, b). Such similarity in organization of the terminal domains of viruses with segmented RNA genomes provides evidence that both the conserved termini and the segment-specific inverted repeats could play a role in determining the secondary structure of the mRNA which could in turn be important in the sorting and assembly of these genomes (Anzola *et al.* 1987).

```
5'     GU UU A AUUCAC C AUGG .....
3'  CAUUC AA C UAAGUG UACC .....
```

Figure 2.11 Inverted repeat sequences of AHSV-3 segment 2 termini.

In comparative analyses with analogous orbiviral genome segments (Table 2.5), genome segment 2 of AHSV-3 was found to be similar in composition to AHSV-4, EHDV-1 and BTV and in length to AHSV-4. However, the AHSV VP2-encoding genes are approximately 250 to 300 bp longer than those of EHDV and BTV. Best-fit alignments of the segment 2 base sequences of AHSV-3 and AHSV-4 with the DNASTAR lasergene computer program with a gap penalty of 10 revealed a 55.76% identity of nucleotides in type and position (requiring 6 gaps with 20 unmatched bases). This is comparable to the BTV interserotype segment 2 identity, which was found to vary from 45.53% between BTV-13 and BTV-17 (6 gaps, 104 bases) to 68.84% between BTV-10 and BTV-17 (1 gap, 3 bases) in similar analyses.

Table 2.5 Comparative analysis of orbiviral segment 2 nucleotide sequences.

Orbivirus	length (bp)	base composition				non-coding region (bp)		coding region (bp)
		%A	%C	%G	%U	5'	3'	
AHSV-3	3221	31.2	15.1	26.1	27.6	12	35	3174
AHSV-4	3229	31.9	14.4	25.7	28.0	12	34	3183
EHDV-1	2967	34.1	15.2	24.7	26.0	16	35	2916
BTV-10	2926	31.0	17.7	23.7	27.7	19	36	2880

By translation of the AHSV-3 segment 2 nucleotide sequence, the encoded VP2 protein was deduced to comprise 1057 amino acid residues with a predicted M_r of 123,078 kDa. This is similar to the 123,945 kDa 1060 amino acid AHSV-4 VP2 published by Iwata *et al.* (1992).

Interserotype comparisons of the available orbiviral VP2 amino acid sequences were carried out by matrix comparison (Fig 2.12) using the Microgenie™ sequence analysis program (Beckman; Queen & Korn 1984) with the default parameters (i.e. minmatch and minper values of 6 and 75 respectively), and by alignment (Fig 2.13) using the Clustal V computer program (Higgins *et al.* 1992) with the default parameters (i.e. fixed and floating gap penalties of 10). Best-fit alignments of the VP2 proteins of AHSV-3 and AHSV-4 required 6 gaps involving 11 amino acids and revealed a 50.5% identity of aligned amino acids. When amino acids of similar character were taken into consideration, this index increased to 71.3%. Interserotype comparisons of BTV VP2 proteins yielded amino acid identities (and similarities) varying from 38.0% (66.4%) between BTV-3 and BTV-10 to 72.4% (88.2%) between BTV-11 and BTV-17. These best-fit alignments required 12 gaps involving 21 amino acids and 1 gap involving one amino acid respectively.

A graphic representation of the best-fit alignment of VP2 of AHSV-3 and AHSV-4, indicating the regional percentage of identical amino acids, is shown in Fig 2.14A, along with the corresponding hydropathicity profiles in Fig 2.14B. The amino and carboxy termini were the most highly conserved regions, with approximately 67% identical amino acids. Two variable regions, with 34% to 36% identity of amino acids, were identified adjacent to the termini. The hydropathicity profiles exhibited significant similarity, revealing strong hydrophilic characters common to all orbiviral outer capsid proteins (Iwata *et al.* 1992), with a few regions of hydrophobicity evident particularly toward the C-terminus. The variability associated with the distinctively hydrophilic domain between amino acids 128 and 309 also seen in the matrix comparison suggests that the determinant(s) of the serotype-specific immune response on VP2 may be located in this region within the overall structurally conserved antigen.

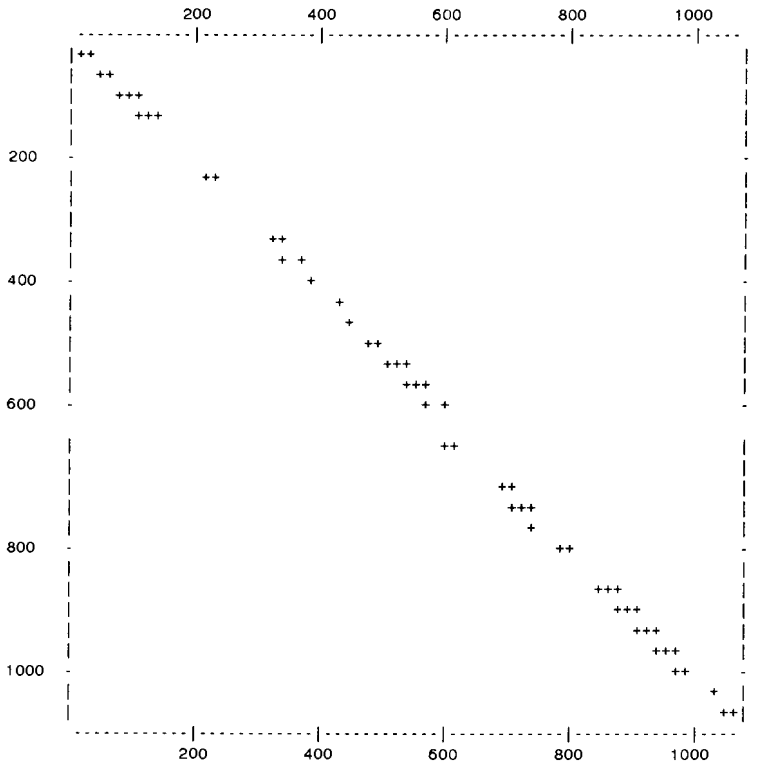

Figure 2.12 Matrix comparison of the VP2 amino acid sequences of AHSV-3 (horizontal axis) and AHSV-4 (vertical axis).

```
MASEFGILLTNQIYDQTYEKEMCDVIITAENAVRRVEVAGVHGYEWGATNHRLGLCEVENTKSIGRMIYEQIRCEGAYPIFPHYITDTLKYGKSIDRNDNQIRVDRDDERVRKIKIQPYF  120
MASEFGILMTNEKFDPSLEKTICDVIVTKKGRVKHKEVDGVCGYEWDETNHRFGLCEVEHDMSISEFMYNEIRCEGAYPIFPRYIIDTLKYEKFIDRNDHQIRVDRDDNEMTKILIQPYA  120

GEMYFSPENYITVFCKRQAISGQIEVSRSIIGRRMKYEESAEQTKGTINANKYRLLEKWRDLAYEQI-EIERDNERCLTHNTDPIYQLIKKMRYGMMYPVHYMLNDRYKVVQERADMGVE  239
GEMYFSPECYPSVFLRREARSQKLDRIRNYIGKRVEFYEEESKRKAILDQNKMSKVEQWRDAVNERIVSIEPKRGECYDHGTDIIYQFIKKLRFGMMYPHYYVLHSDYCIVPNKGGTSIG  240

KWLLQKIGRGTQRRKADDGDNDALLQLERMMSSEELERSVIESVIRFGSLYNAHAGKKTGDIPLEVLIKYCDSLTTFVHKKNREGGDNQTARDEIRRAVVKNIPSMKQENQMKVTPNI-R  358
SWHIRKRTEGDAKASAMYSGKGPLNDLRVKIERDDLSRETIIQIIEYGKKFNSSAGDKQGNISIEKLVEYCDFLTTFVHAKKKEEGEDDTARQEIRKAWVKGMPYKDFSKPMKITRGFNR  360

NFLFFAYLNGFKRNNGVDIDPNNGTWSKHKAEVKKFLNEEQKKNENKPLKVLIDGAYISTDAEYGTVAHWVDWVVDIVMMTQVSRMIKEYNFIRLKKDQLISGMNKLEDGVKCYAYCLIL  478
NMLFFAALDSFRKRNGVDVDPNKGKWKEHIKEVTEKLKKAQTENGGQPCQVSIDGVNVLTNVDYGTVNHWIDWVTDIIMVVQTKRLVKEYAFKKLKSENLLAGMNSLVGVLRCYMYCLAL  480

ALYDFHGRDVDGFAQQGTRTAAIVETVARMFPDFRSEVSEKFGIDLAVSEESDELFVKKTMVSSFSDSGEMGYKFIFGWRKTDFKVETDYGEIVSDEVHRLYQAILDGKEWSKEVDDPEKY  598
AIYDFYEGTIDGFKKGSNASAIIETVAQMFPDFRRELVEKFGIDLRMKEITRELFVGKSMTSKFMEEGGYGYKFAYGWRRDGFAVMEDYGEILTEKVEDLYKGVLLGRKWEDEVDDPESY  600

FVDDLYNRCPESIYVRNGVDPNNKIMIKKRGLVGESQRIFLRDLSHIGMNFKKLLLRLSSKR---LHARGEHIQYHEIDVEDFKPCAIAELGLHCSTYIYQDLLVGANRGEYVKDAKELV  715
FYDDLYTNEPHRVFLSAGKDVDNNITLRS---ISQAETTYLSK-RFVSYWYRISQVEVTKARNEVLDMNEKQKPYFEFEYDDFKPCSIGELGIHASTYIYQNLLVGRNRGEEILDSKELV  716

WFDIANTNFNITRPFDRCWPSSCAEAELSLRFHLITKIFTRY--RGERTSFVDIINELSEHGYVKHNFPSYKHYYLSVIQTVFEDQRAIDPLDFCAMISRNETRESTLKGFSMFTAIVKS  833
WMDMSLLNFGAVRSHDRCWISSSVAIEVNLRHALIVRIFSRFDMMSERETFSTILEKVMEDVKKLRFFPTYRHYYLETLQRVFNDERRLEVDDFYMRLYDVQTREQALNTFTDFHRCVES  836

ERLIDTLFLNFLLWIVFEMENVDVSAANKRHPLLISHEKGLRLIGVDLFNGALSISTGGWIPYLERICSEEKAQRRLNADELKIKSWFLTYYMNLSLERRAEPRMSFKFEGLTTWIGSNC  953
ELLLPTLKLNFLLWIVFEMENVEVNAAYKRHPLLISTAKGLRVIGVDIFNSQLSISMSGWIPYVERMCAESKVQTKLTADELKLKRWFISYYTTLKLDRRAEPRMSFKFEGLSTWIGSNC  956

GGVRDYVVQALPMRKPKPGLLMIIYGDDGDARWVEWAMKNFTAVDGSLGFIYIDRHKLVNKSDFRVREMKIYNRGRLDRLILISSGHYTFGNKFLMSKLLAKTE  1057
GGVRDYVIQMLPTRKPKPGALMVVYARDSRIEWIEAELSQWLQMEGSLGLILVHDSGIINKSVLRARTLKIYNRGSMDTLILISSGVYTFGNKFLLSKLLAKTE  1060
```

Figure 2.13 Best-fit alignment of the amino acid sequences of the VP2 proteins of AHSV-3 (top) and AHSV-4 (bottom). The * and . symbols denote identical and similar amino acid residues respectively.

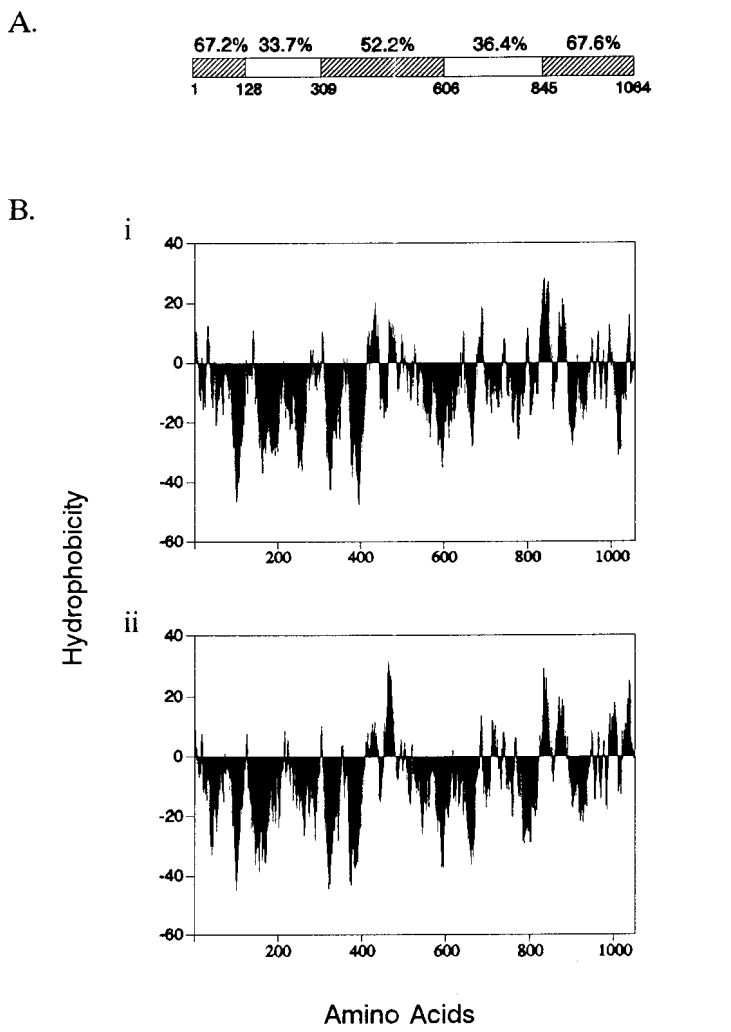

Figure 2.14 A graphic representation of the best-fit alignment of the amino acid sequences of VP2 of AHSV-3 and AHSV-4 (A), indicating the regional percentage identical amino acids (A), and the corresponding hydropathicity profiles (Kyte & Doolittle 1982) (B) of VP2 of AHSV-3 (i) and AHSV-4 (ii).

A phylogenetic analysis of the orbiviruses based on the analogous VP2 proteins sequenced to date was carried out using the Clustal V computer program by multiple alignment of the proteins and the generation of a phylogenetic tree based thereon. Large numbers of gaps were required to effect the multiple alignment of the VP2 proteins and little overall amino acid identity or similarity was revealed. This lack of similarity was also reflected in interserogroup matrix comparisons carried out using the Microgenie™ program (data not shown). By way of illustration, the alignment of only the AHSV-3, EHDV-1 and BTV-10 VP2 sequences produced with the Clustal V program and the default parameters is shown in Fig 2.15. The regions that exhibited the greatest similarity occurred near the carboxy terminus, few unmatched amino acids being necessary to optimally align the approximately 270 carboxy-terminal residues. The results in terms of the percentage of identical and similar amino acids in type and position and the number of gaps required in the aligned sequences are given in Table 2.6.

```
MASEFGILLTNQIYDQTYEKEMCDVIITAENAVRRVEVAGVHGYEWGATNHRLGLCEVENTKSIGRMIYEQIRCEGAYPIFPHYITDTLK-YG-KSIDRNDNQIRVDRDDERVR-----K  113
ME-DINLTIFSDD-ALPHAVVVDDYVIAIKRNPNGTFLEPHQMYDRYTQEF---LQGKAR----DVLRQNGRDGYKL--IIPEALSTGIIRYENKTKGAQSE-VELENQ---LRSSIRH-  104
ME-EFVIPVFSER-DIPYSLL-NHYPLAIQIDVKVDDEGGKHNLIKIPESD---MIDVPRLSIIEALNYRPKRNDGV--VVPRLLDITLRAYDNRKSAKNAKGVEFMTDTKWMKWAIDDK  112
*  . . . . .  .  . .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .

IKIQPYFGEMYFSPENYITVFCKRQAISGQIEVSRSIIGRRMKYEESAEQTKGTINANKYRLLEKWRDLAYEQIEIERDNERCLTHNTDPIYQLIKKMRYGMMYPVHYMLNDRYKVV---  230
---QRV----------------KPRMDEAHRKLQIELRGGQILLHPRIAES-----------------IEFSIISKENAT--CSHTPVNCAYEVLLSGGINVGTGTCYDLSSRLKLRVIG  186
MDIQPL----------------KVTLDN-HCSVNHQLFNCIVKARSANADT-----------------IYYDYYPLENGAKRCNHTNLDLLRSLTTTEMFHILQGAAYALKTTYEL--VA  196
   *                    *     .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .

-QERADMGVEKWLLQKIGRGTQRR---KADDGDNDALLQLERMMSSEELERSVIESVIRFGSLYNAHAGKKT-GDIPLEVLIKYCDSLTTFVHKKNREGGDNQTAR--DEIRRAVVKNIP  343
DVDRHRRSMQN-VLGRVIHTGDPKIINRVNQIGSQQFIDRAIGPDKFELKREIFDRLK--A--LDVDVRKVIREE--EAS-----AELDEMGRRWMRD--QNVNIVN-DIIQSLVKKGSR  291
HSEREN-----------------MSESYQVGTQRWIQLRKGT-KIGYRGQPYERFI--SSLVQVIIKGKIPDEIRTEI-----AELNRIKDEWKNAAYDRTEIRALELCKILSAIGRK  289
  .*                  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .

SMKQENQMKVTPNIRNFLFFAYLNGFKRNNGVDIDPNNGTWSKHKAEVKKFLNEEQ-----------KKNENKPLKVLIDGAYISTDAEYGTVAHWVDWVVDIVMMTQVSRMIKEYNFI  451
SEKLAH--RNEQGMQARFRRTIATNL-RDQRQGKEVLNIRGTRGQPEEKKFAAVLLMTGCDIVERAIWSNEETAILRGLYAYAHDKLGCVYRAMKKDFVWSIRPTYTDRCAGVCDRKRTI  408
MLDVQEEPKDEMALSTRFQFKLDEKFIRTDQEHVNIFKVGGS--ATDDGRFYALIAIAGTDTQQGRVWRTNPYPCLRGALIAAECELGDVYFTLRQTYKWSLRPEYGQRERPL-EDNKYV  406
   .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .

RLKKDQLISGMNKLEDGVKCYAYCLILALYDFHGRDVDGFAQGTRTAAIVETVARMFPDFRSEVSEKFGIDLAVSEESDELFVKKTMVSSFSDSGEMGYKFIFGWRKTDFKVETDYGEIV  571
-MVREDYFDLQREENDSV----YKWIITEWD-----------KNDVIISAKNGYLYSKYSGEDED----DILVHEIDDRLY--TAMIDRI---------LINGWIEKEGLS-----QII  491
-FARLNLFDTNLAVGDEI----IHWRYEVYQ-----------PKE--TTHDDGYICVSQKGDDE-------LLCEVDEDRY--KEMFDRM---------IQGGWDQERFKL-----HNI  484
   .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .

SDEVHRLYQAILDGKEWSKE--VDDPEKYFVDDLYNR-CPESIYVRNGVDPNNKIMIKKRGLVGESQRIFLRDLSHIGMN--FKKLLLRLSSKRLHARGEHIQYHEIDVEDFKPCAIAEL  686
KEEVR------LESFDFTKDAYVDEAGFLVLPEYYDRVIASNIY--DCKFKISRVSITSSSNDDPWDKKTADSIIDE--QCLWKIPLPNIIDVRPCFRGDLLTSNSQEYSKRFSGIIDEL  601
LTEPN------LLTIDFEKDAYLGARSELVFPPYYYDKWINSPMF--NARLKIARGEIATWKADDPWSNRAVHGYIKTSAESL-EYALGPYYDLRLQLFGDTLS-----LGQRQSAVFEHM  590
   *     *  .  *.  .  .  *..  .  .  .  .  .  *  .  .  .  .  .  .  .  *  .  .  *  *. .  .  .  .  .  .  .  .  .  .  .  .

GLHCSTYIYQDLLVGANRGEYVKDAKELVWFDIANTNFNITRPFDRCWP-----SSCAEAELSLRFHLITKIFTRYRG--ERTSFVDIINELSEHGYVKHNFPSYKHYYLSVIQTVFEDQ  799
KKDKEIY---DDFIPVQEGVRPCVQGHVCRYAFYRQKLTIFTILKRYYPIERILELTDEEDYEYNLYLDKECYKKESLILNLRSIFSLICFLIDFGYEGREITRGEDEYLKIFNEI----  714
AQQDD-F---STLTDYTKGRTVCPHSGGTFYTFRKVAL---IILSNYERLDPSLHEGREHE-TYMHPAVNDVFRRH--VLEMKDFSQLICFVFDYIFEKHVQLRNAKEARRIIYLI----  696
   .  .  .  .  *  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .

RAIDPLDFCAMISRNETRESTLKGF-SMFTAIVKSERLIDTLFLNFLLWIVFEMENVDVSAANKRHPLLISHEKGLRLIGVDLFNGALSISTGGWIPYLERICSEEKAQRRLNADELK--  916
------NY-GGHARKEAINKYFPQFYQRLMRVRTSENIEDLLPLAFYQALLLSDPCTDNSEKS-SHPLILFCQDKVRVVPIRTATQERGLPLLCCIHIF-KFHPGLQMRKKELEDDIKKT  825
------QNTSGAYRLDVLREAFPNFLKHVMNLRDVKRICDLNVINFFPLLFLVQDNISYWHRQWSIPMILF-DQVIRLIPVEVGAYANRFGLKSFFNFI-RFHPG-DSKKRQDADDTHKE  807
      .  .  *  .  .  .  *  .  .  .  *  .  *  .  .  .  .  .  .  .  .  .  .  *  .  .  .  *  .  .  .  .  .  .  .  .  .  .  .  .

IKSWFLTYYMNLSLER--RAEPRMSFKFEGLTTWIGSNCGGVRDYVVQALPMRKPKPGLLMIIYGDDG--DARWVEWAMKNFTAVDGSLGFIY---IDRHKLVNKSDFRVREMKIYNRGR 1029
LPAI-FDYWIELEMKRLDTGDRLRT-RAQMVELYYSTNCGGSYETLNFVFPIVHPNKGFIACVISSKGGMGALNEDDVRRRFRRIQSSIQGIFSISIDEEMEIQLHHSGNIQARILEKVF  943
FGSICFEYYTTTKISQGEIDVPVVTSKLDTLKLHVASLCAGLADSLVYTLPVAHPKKSIVLIIVGDDKLEPQIRSEQIVNKYYYSRRHISGVVSICVNQGGQLKVHSMGITRHRICDKSI  927
. . . *  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .

LD---RLILISSGHYTFGNKFLMSKLLAKTE  1057
FEHKWHIVQVKLNGKIFENHELITKLMN---   971
LKYKCKVVLVRMPGHVFGNDELMTKLLNV--   956
.    . . .      * *  *..**.
```

Figure 2.15 Best-fit alignment of the amino acid sequences of VP2 of AHSV-3 (top), EHDV-1 (middle) and BTV-10 (bottom). Symbols are as in Fig. 2.13.

Table 2.6 Identity and similarity of the amino acid sequences of VP2 of AHSV-3, BTV-10 and EHDV-1 based on best-fit alignments.

	AHSV-3 / BTV-10	AHSV-3 / EHDV-1	BTV-10 / EHDV-1
% identity	16.85	15.24	21.26
% similarity	40.09	41.21	51.90
number of gaps (amino acids)	49 (207)	46 (190)	34 (77)

A striking feature of the VP2 proteins is the proportion of conserved cys, pro and gly residues, accounting for 8 of the 19 identical amino acids in the multiple interserogroup alignment. These residues are likely to be required for maintaining the folding pattern of the proteins.

The phylogenetic tree obtained using the default parameters of Clustal V, portraying the orbiviral interserogroup relationships derived from the alignment of the VP2 amino acid sequences, is shown in Fig 2.16. The AHSV, BTV and EHDV serogroups are clearly delineated as distinct branches, EHDV being more closely related to BTV than to AHSV. The score of the most distant pair is 0.91 (EHDV-1 and AHSV-4) while the scores of the most distant and closest intraserogroup pairs are 0.62 (BTV-10 and BTV-3 or BTV-13) and 0.28 (BTV-11 and BTV-17) respectively.

Comparisons of the hydropathic profiles of the selected orbiviruses (Fig 2.17) also reflects the lack of interserogroup similarity. Nevertheless, based on overall composition (Table 2.7) and hydrophilicity, the predicted sequences of the VP2 proteins of all three orbiviruses are clearly related at the structural level.

However, analysis of the VP2 amino acid sequences of AHSV and other orbiviruses can at best merely provide an indication of the function and antigenicity of the protein and its domains. In order for a more in-depth study of these aspects to be carried out, particularly with a view to the development of efficacious subunit vaccines, it was necessary to obtain sufficient quantities of the protein in its native form.

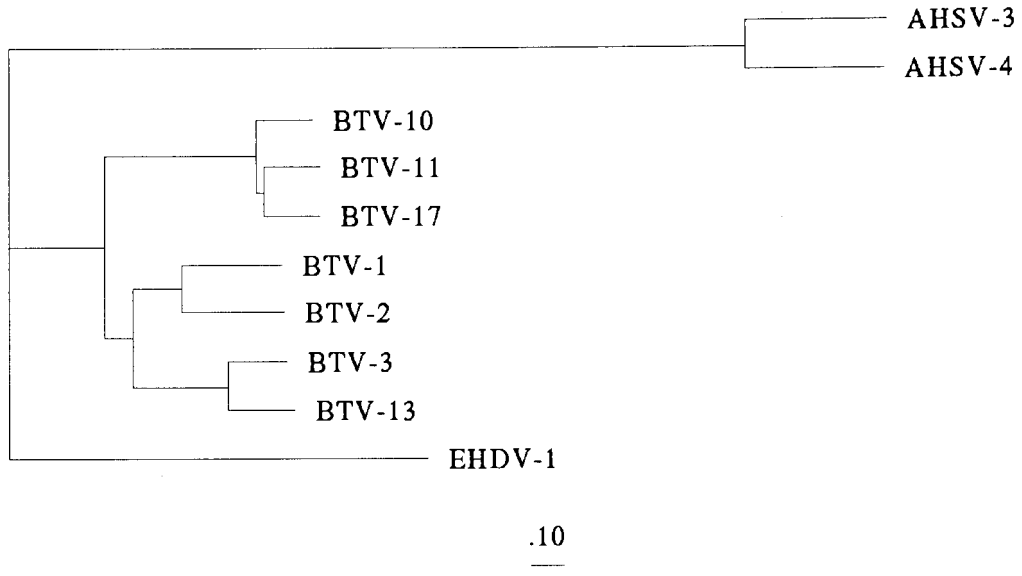

Figure 2.16 A phylogenetic tree depicting the relationship between various orbiviral serogroups based on a comparison by amino acid sequence alignment of the VP2 proteins. EHDV-1 VP2 was used as an outgroup. A scale bar for the branch lengths is indicated.

Table 2.7 Comparisons of the length, amino acid composition, charge and molecular weight of the VP2 proteins of AHSV-3, AHSV-4, EHDV-1 and BTV-10.

	AHSV-3	AHSV-4	EHDV-1	BTV-10
Length (amino acids)	1057	1060	971	956
% Acidic (D,E)	14.3	14.8	15.4	13.5
% Basic (R,K,H)	16.8	16.6	15.6	14.7
% Aromatic (F,W,Y)	11.0	11.8	9.3	10.8
% Hydrophobic (A,I,L,F,W,V)	35.9	36.9	34.9	36.1
Charge	+15	+9.5	+4	+11.5
Molecular weight (kDa)	123,078	123,945	113,261	111,034

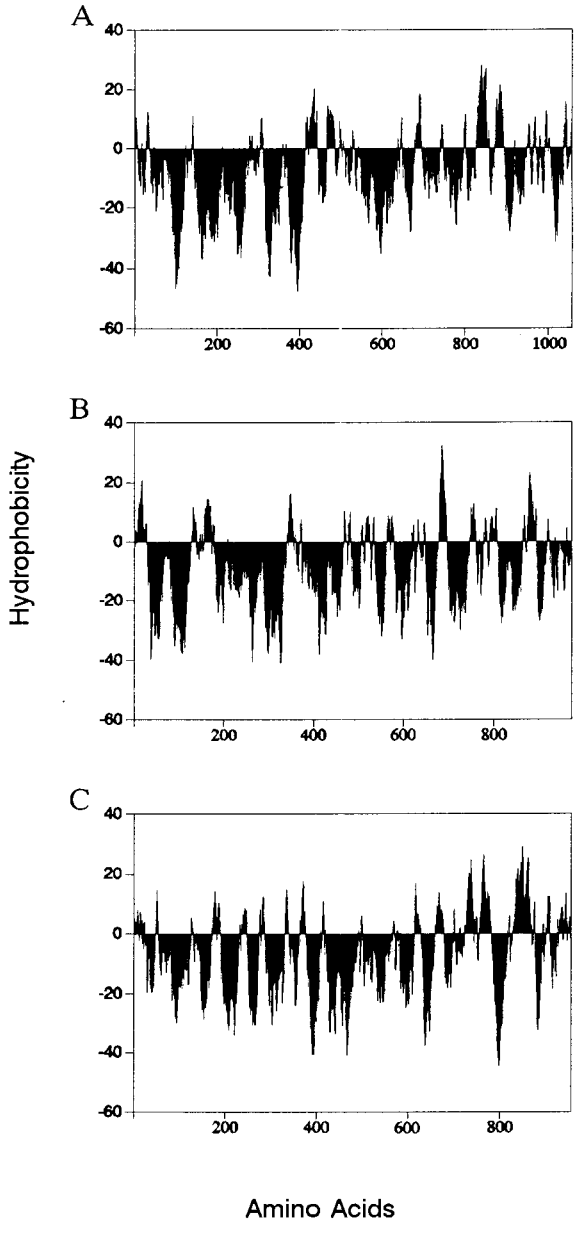

Figure 2.17 Comparisons of the hydropathicity profiles (Kyte & Doolittle 1982) of VP2 of AHSV-3 (A), EHDV-1 (B) and BTV-10 (C), showing regions with a net hydrophobicity (positive values) and a net hydrophilicity (negative values) using a window size of 21.

CHAPTER 3

EXPRESSION OF THE VP2 PROTEIN OF AFRICAN HORSESICKNESS VIRUS SEROTYPE 3

3.1 INTRODUCTION

Recent advances in gene manipulations have made it possible to express foreign genes in heterologous systems. In general, the cloning and subsequent expression of individual proteins can assist in the elucidation of the function of each protein in virus structure, replication and assembly. Analysis of the antigenic, functional and molecular properties of expressed gene products, alone or in conjunction with other viral proteins, can also allow intrinsic properties of each gene product to be determined. In addition, humoral and cell-mediated immune response(s) to specific viral proteins can be more easily and directly dissected with the availability of high levels of individual proteins. Finally, the availability of large amounts of the immunogenic structural proteins will facilitate vaccine testing and the production of inexpensive diagnostic tests.

It has been demonstrated that VP2 bears neutralizing epitopes for AHSV and that antibodies to these epitopes are protective *in vivo* (Burrage *et al.* 1993). This suggests that recombinant VP2 expressing these epitopes may have efficacy as a subunit vaccine against AHSV. In the case of BTV, VP2 recovered from purified virus (Huismans *et al.* 1987b) or derived from baculovirus expression vectors (Roy *et al.* 1990) has been demonstrated to induce neutralizing antibodies and to elicit protection in sheep against virulent viral challenge. VP5 was found to enhance the neutralization (and protective) immune response (Roy *et al.* 1990). Recently considerable effort has been exerted in the production, through baculovirus-mediated expression, of the major capsid proteins, and evaluation of VLPs as subunit vaccines against BTV (Van Dijk 1993). As no similar data was available for AHSV VP2, it was decided to express a cloned copy of the AHSV-3 segment 2 gene in a eukaryotic expression system, with a view to performing an in depth functional and immunological analysis of the expressed protein. A eukaryotic expression system offers the advantage over prokaryotic systems of producing large amounts of the foreign, in this case viral, protein in a near to native form.

As described in the previous chapter, a full length copy of the AHSV-3 segment 2 gene was cloned, and characterized by sequencing. The amino acid sequence of the encoded VP2 was deduced by translation and analysed. This chapter describes the cloning and expression of the AHSV-3 gene in the baculovirus expression system, while allowing for the possible future coexpression of other capsid proteins.

3.2 MATERIALS AND METHODS

3.2.1 Insertion of AHSV-3 segment 2 into a baculovirus transfer vector

All gene manipulation techniques were essentially as described in Materials and Methods in Chapter 2.

Partial BglII digestion of pAHSV3.2

The recombinant pBR322 plasmid, pAHSV3.2 (2.3.5), contains a full length copy of the AHSV-3 segment 2 gene flanked by BglII restriction sites, introduced by the primers during PCR amplification, and by homopolymer dG/dC tails, introduced as a result of the cloning strategy used. Full length AHSV-3 segment 2 suitable for cloning in a baculovirus transfer vector was obtained by performing a partial BglII digestion of pAHSV3.2 as described by Ausubel *et al.* (1988). 2µg pAHSV3.2 was diluted to 60µl in the recommended salt buffer and aliquotted to nine Eppendorf tubes in 1 x 12µl and 8 x 6µl quantities. 0.5µl BglII (11 units/µl) was added to the 12µl aliquot on ice and then 6µl of the reaction mix was consecutively transferred to successive tubes in the series. 6µl from the final tube was discarded. The reactions were incubated for 1 h at 37°C and then analysed by 0.8% 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 3.2 kb AHSV-3 segment 2, was calculated and the reaction was scaled up as follows: 1µg pAHSV3.2 was incubated with 0.4 units BglII in the recommended salt buffer in a final volume of 18µl for 1 h at 37°C. The 3.2 kb DNA bands representing AHSV-3 segment 2 were excised from the gel and purified by Geneclean™ extraction.

Recloning of AHSV-3 segment 2 into pAcUW3

The baculovirus dual expression transfer vector, pAcUW3 (Fig 3.1), was obtained as a gift from the NERC Institute of Virology, Mansfield Road, Oxford, UK. Plasmid pAcUW3 was linearized with BglII, dephosphorylated and ligated to purified full length BglII-restricted AHSV-3 segment 2 DNA. After transformation into competent *E. coli* Xl1-blue cells and plating out on LB agar plates containing amp and tet, plasmids from a number of the colonies obtained were isolated and characterized by restriction analysis. Recombinant plasmids with the correct transcriptional orientation of segment 2 relative to the p10 promoter were selected by BamH1 restriction mapping. A large scale extraction of this recombinant transfer vector, designated pAcAHSV3.2, was followed by CsCl gradient purification of the plasmid.

3.2.2 Cells and viruses

The origin of AHSV-3 and its propagation in a Vero cell line were as described in 2.2.1. *LacZ* recombinant *Autographa californica* nuclear polyhedrosis virus (AcRP23-*lacZ*), derived by Possee & Howard (1987), and *Spodoptera frugiperda* (Sf9) cells were obtained from the NERC Institute of Virology, Mansfield Road, Oxford, UK. All cell culture handling techniques were essentially as described by Summers & Smith (1987). The Sf9 cells were grown as confluent monolayers or as suspension cultures in spinner flasks at 28°C in Grace's insect medium modified with 3.3g/l yeastolate and 3.3g/l lactalbumin hydrolysate (Highveld Biological) to which 10% foetal calf serum (FCS) and antibiotics had been added. Cell density of suspension cultures were determined using a

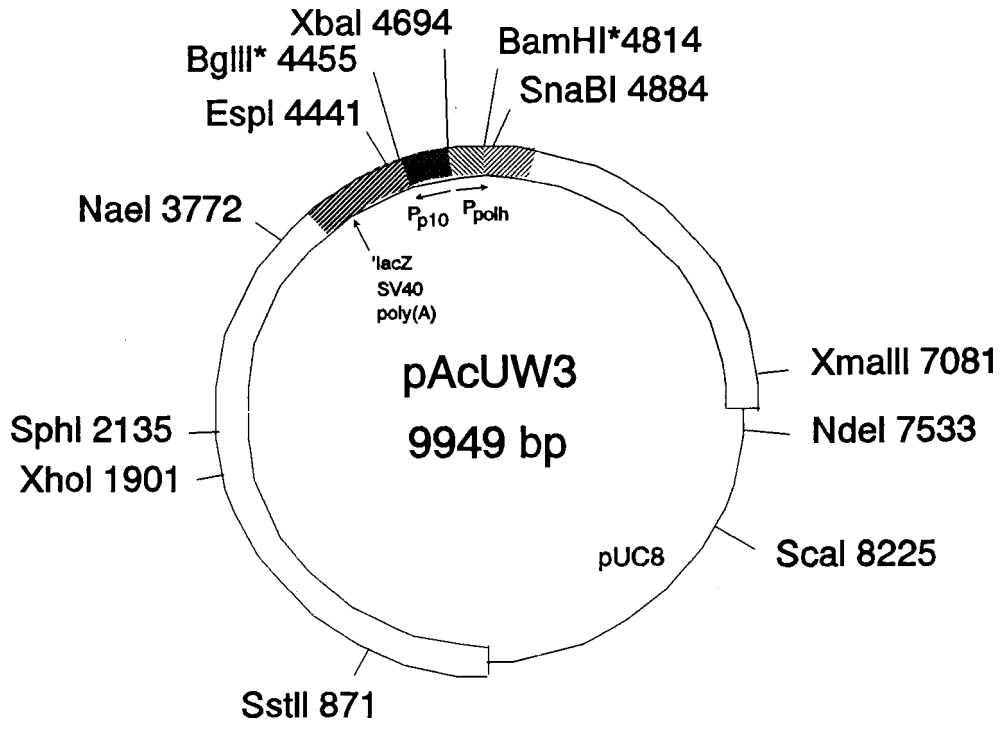

Figure 3.1 A restriction map of pAcUW3 indicating the positions of the polyhedrin and p10 promoters. The arrows indicate the orientation of the promoters, and the asterisks mark the unique BamH1 and BglII cloning sites for the insertion of foreign genes.

haemacytometer, the addition of trypan blue serving to inspect cell viability; cells taking up trypan blue were considered nonviable. The cultures were seeded at an initial density of 0.2-0.5 x 10^6 cells/ml in a minimum of 50 ml and subcultured when a density of 2-2.4 x 10^6 cells/ml was attained.

Suspension cell cultures were infected with virus by pelleting of the appropriate volume of cells by centrifugation at 1500 rpm in a Beckman JS7.5 rotor for 10 min, resuspension in 1/10 of the original volume Grace's medium supplemented with virus at a MOI of 0.1 pfu/cell. Following incubation at RT for 1-2 h, the culture was diluted to a density of 1-1.5 x 10^6 cells/ml with Grace's medium and incubated further with spinning at 28°C for 2-4 days.

Virus infection of monolayers was carried out by seeding of cells into flasks or plates at a density of approximately 1.5-1.8 x 10^5 cells/cm^2 and allowing to attach for 1-2 h prior to removal of the medium and the addition of virus to a MOI of 1 pfu/cell. After 1-2 h at RT, the inoculum was replaced with fresh medium. The cells were incubated at 28°C for 2-4 days.

3.2.3 Production of VP2 recombinant baculovirus

Purification of extracellular baculovirus DNA

During AcNPV infection, extracellular virus is released from the cell by budding. Relatively pure viral DNA was obtained from extracellular virus particles, separated from infected cell culture

medium by centrifugation, as described by Summers & Smith (1987). The culture medium of a 100ml suspension culture of Sf9 cells (1.6 x 10^6 cells/ml) infected with AcRP23-*lacZ* was harvested at 48-72 h pi by centrifugation at 3000 rpm in a Beckman JS7.5 rotor for 10 min. Virus was collected from the supernatant by centrifugation at 25000 rpm for 60 min in a Beckman SW28 rotor and the pellet was resuspended in 2-3ml 0.1x TE. The virus was purified on a step sucrose gradient prepared from equal volumes 50% and 5% sucrose in 0.1x TE and centrifuged at 24000 rpm for 60 min in a Beckman SW28 rotor. The diffuse white band of virus particles at the interphase was removed with a glass syringe, diluted to 30ml with 0.1x TE and precipitated as before. After resuspending the pellet in 800 µl 0.1x TE, the DNA was isolated by the addition of 200 µl 10% sarkosyl and incubated at 60°C for 15 min. Care was taken to prevent degradation of the viral DNA by mechanical shearing. The cleared suspension was diluted with 3ml 0.15M STE and 200 µl 1M Tris-HCl pH8.5 and extracted once with 4ml phenol/chloroform/isoamylalcohol. The supernatant was collected by centrifugation at 7000 rpm for 5 min and the interphase was back extracted with 1ml 0.15M STE. The collective supernatant was extracted once with chloroform and the DNA precipitated at -20°C by the addition of 2 volumes 100% ethanol. After centrifugation at 2500 rpm for 20 min and washing with 96% ethanol, the DNA was dried slightly, resuspended in 0.1x TE and stored at 4°C.

Linearization of AcRP23-*lacZ* DNA

1 µg of AcRP23-*lacZ* DNA was incubated with 5 units Bsu36I in the appropriate buffer for 2 h at 37°C. The enzyme was inactivated by heating to 70°C for 15 min. The extent of digestion was monitored by 0.3% agarose gel electrophoresis at 40V for 3 h.

Cotransfection of Sf9 cells

Sf9 cells were cotransfected with linearized AcRP23-*lacZ* DNA and the recombinant baculovirus transfer vector plasmid by lipofection. This procedure 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). Sf9 cells were seeded in 35mm wells as described and washed twice with Grace's medium without FCS before adding a further 1ml FCS-free medium. 2 µg CsCl-purified recombinant transfer vector plasmid was mixed with 1 µg linearised AcRP23-*lacZ* DNA in a volume of 17.5 µl and 12.5 µl lipofectin™ reagent (BRL) was diluted to 17.5 µl in a separate tube. The two solutions were mixed and added dropwise to the cells. After an incubation period of 5-24 h at 28°C under humified conditions, the medium was replaced with 2ml medium containing FCS and the incubation was continued. From 72 h post transfection, 1ml samples of the medium were taken on three consecutive days for titration, a further 1ml medium being added on the second day.

Virus titration and plaque purification

Cells were seeded in 35mm wells as described in 3.2.2. Dilution series of the transfection supernatants, from 1.5 x 10^{-1} to 1.5 x 10^{-6} in 1ml medium, were prepared and added to the cells. After 1-2 h at RT, the inoculum in each well was replaced with 2ml 3% low melting agarose diluted 50% with Grace's insect medium at 37°C. Following an incubation period of 4 days at 28°C, 1ml Neutral Red (0.1mg/ml in Grace's medium) containing 250 µg/ml X-gal was added to each well and left for 5-7 h at 28°C. The following day, the wells were screened for transparent patches in the red background, representing plaques caused by recombinant baculovirus, as opposed to wild-type baculovirus-infected

cells which were visible as blue plaques.

Recombinant plaques were picked as agarose plugs using a 1ml pipette and sterile tips with shortened ends, and deposited in 1ml Grace's medium. Following vigorous vortexing to release the virus (approximately 10^4 pfu/plug), a 2 x 10^{-3} dilution of each putative recombinant was titrated as described above. Following dot hybridization of recombinant baculovirus DNA, selected positive recombinants were subjected to two rounds of plaque purification.

3.2.4 Analysis of recombinant baculovirus nucleic acids

Dot hybridization of recombinant baculoviruses

Approximately 2 x 10^3 pfu of each potential recombinant virus obtained in 3.2.3 was used to infect Sf9 cells seeded in a 24-well (16mm) tissue culture plate. After 2-3 days at 28°C in humidifed conditions, the medium was removed and the cells were lysed by the addition of 1ml 0.5N NaOH. The solution was neutralized by the addition of 140µl 7.5M NH_4Ac and 200µl of each lysate spotted onto Hybond N+ nylon membrane equilibrated in 1M NH_4Ac, 0.2N NaOH using a dotblot apparatus (Biorad). The membrane was rinsed for 2 min in 2x SSC and the DNA fixed by UV irradiation for 5 min. The membrane was probed with radiolabelled pAHSV3.2 by hybridization and autoradiography.

Detection of viral mRNA in infected cells

Sf9 cells were seeded in a 24 well plate and infected with recombinant virus at a MOI of 10 pfu/cell. The cells were harvested at approximately 6 hourly intervals from 0 to 46 h pi by resuspension in the supernatant using a micropipette and were then immediately frozen at -20°C until further use. The following procedure to detect viral RNA *in situ* was as described by Paeratakul *et al.* (1988). Samples of 1 x 10^5 cells representing each time interval were spotted onto Hybond C-extra nitrocellulose membrane (Amersham) using a dot-blot microfiltration apparatus. The membrane was fixed in 3% NaCl, 10mM NaH_2PO_4, 40mM Na_2HPO_4, 1% gluteraldehyde for 1 h at 4°C, and then rinsed 3 times with proteolytic buffer (50mM EDTA, 0.1M Tris pH8.0). The fixed cells were digested with 20µg/ml proteinase K in proteolytic buffer at 37°C for 30 min. After baking the filter at 80°C for 1 h, it was prehybridized and hybridized to a segment 2-specific DNA probe.

3.2.5 Analysis of recombinant baculovirus-expressed VP2

Radiolabelling and SDS-PAGE analysis of viral proteins

Monolayers of Sf9 cells seeded in 16mm wells were infected with wild type AcRP23-*lacZ* or recombinant viruses at a MOI of 10 pfu/cell to ensure synchronized infection. After 1 h, the inoculum was replaced with Grace's medium and incubation at 28°C was continued for 26 h. The medium was replaced with 500µl methionine-free Eagle's medium (as methionine-free Grace's medium was not available) and incubated at 28°C for 1 h to deplete intracellular pools of methionine. The starving medium was replaced with 200µl fresh methionine-free Eagle's medium to which approximately 10µCi ^{35}S-methionine per well had been added. The cells were harvested after a 3 h incubation period at 28°C by resuspension in 1ml PBS with a pipette, pelleted by centrifugation followed by disruption of the cell pellet by resuspension in 100µl protein solvent buffer (PSB; 0.125M Tris-HCl pH6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol). For the preparation of radiolabelled AHSV-3 viral proteins, 80cm^2

monolayers of Vero cells grown in Eagle's medium containing 6% FCS were rinsed with serum-free Eagle's medium and infected with 3ml AHSV-3 inoculum with a high titre. After 1 h at 37°C, 3ml additional serum-free Eagle's medium was added and incubated overnight at 32°C. At 17 h pi, the virus was removed and replaced with 2ml methionine-free Eagle's medium. After 1 h at 32°C, the medium was replaced with 1.5ml fresh methionine-free Eagle's medium and approximately 30µCi ^{35}S-methionine was added. After a 3 h incubation at 37°C, the cells were washed in PBS and then resuspended in 100µl lysis buffer (50mM Tris-HCl pH7.5, 150mM NaCl, 1% Triton X-100, 0.1% SDS, 20mM EDTA). Mock infected Sf9 and Vero cells were labelled in a similar manner for use as controls.

SDS-polyacrylamide gel electrophoresis

Prior to electrophoretic analysis, protein samples were mixed with an equal volume 2x PSB, heated to 95°C for 5 min and sonified. The samples were then separated by 10% SDS-PAGE as described by Laemmli (1970). 0.75mm thick separating gels containing 0.375M Tris-HCl pH8.8 and 0.1% SDS and 3% stacking gels containing 0.125M Tris-HCl pH6.8 and 0.1% SDS were prepared from a stock solution of 30% acrylamide, 0.8% bisacrylamide. The gels were polymerized chemically by the addition of 0.008% (v/v) TEMED and 0.08% (m/v) ammonium persulfate. Electrophoresis was carried out in TGS buffer (0.025M Tris-HCl pH8.3, 0.192M glycine, 0.1% SDS) in a Sturdier SE400 vertical slab gel unit (Hoefer Scientific Instruments) for 16 h at 60 V or in a Mighty Small II SE250 unit for 3 h at 125 V. Gels were stained in 0.05% Serva Blue (Coomassie Blue), 50% methanol, 10% acetic acid and destained in 5% methanol, 5% acetic acid at 50°C. In the case of radiolabelled proteins, the gels were dried and autoradiographed overnight as described in 2.2.12.

Western immunoblot analysis

Radiolabelled or unlabelled viral proteins from infected Sf9 or Vero cells were resolved by 10% SDS-PAGE. Thereafter, the gel and a Hybond-C extra nitrocellulose membrane were soaked for 30 min in transfer buffer (48mM Tris-HCl, 39mM Glycine, 20% methanol, pH9.0) and a sandwich was prepared between two sheets of thick filter paper (or six sheets of thin filter paper) presaturated in transfer buffer in a Transblot semi-dry electrophoretic transfer cell (Biorad), with the gel towards the cathode. Transfer was for 1 h at 15 V. The membrane was then washed in PBS for 5 min and incubated in blocking solution (1% m/v fat-free milk powder in PBS) for 30 min at RT after which the primary antibody (rabbit anti AHSV-3 or convalescent horse anti AHSV-4 serum obtained from the OVI and diluted 1/100 in fresh blocking solution) was added and incubated for 1-2 h at RT with agitation or overnight at 4°C. The membrane was washed three times for 5 min in wash buffer (0.05% v/v Tween-20 in PBS) prior to the addition of the secondary antibody (pig anti rabbit IgG conjugated to horseradish peroxidase (Sigma) diluted 1/400 in blocking solution). After incubation for 1 h at RT with agitation, the membrane was washed three times for 5 min in wash buffer and once for 5 min in PBS. The membrane was then transferred to enzyme substrate prepared by dissolving 60mg 4-chloro-1-naphtol in 20ml ice cold methanol and mixing it with 60µl H_2O_2 in 100ml PBS just before use, and incubated at RT until the AHSV-specific bands became visible. The membrane was rinsed in water and air-dried.

3.3 RESULTS

3.3.1 Construction of an AHSV-3 segment 2 baculovirus transfer vector recombinant

Owing to the large size of the baculovirus genome (about 130 kb), recombinant virus construction relies on *in vivo* recombination to replace a viral allele with the gene of interest. Thus, in order to obtain a recombinant baculovirus, the foreign gene has to be cloned into a 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 necessary for homologous recombination. Subsequent cotransfection 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.

A baculovirus dual expression transfer vector, pAcUW3 (Fig 3.1), developed by Weyer & Possee (1991), facilitates the introduction of two heterologous foreign genes inserted at unique BamH1 and BglII sites into a single recombinant virus. Both foreign proteins are expressed, under the control of the very late polyhedrin and p10 promoters respectively.

The strategy employed for the cloning of the full length AHSV-3 VP2 gene has been described in the previous chapter. Briefly, BglII restriction sites were introduced at the 5' and 3' termini of the genome segment 2 cDNA through the PCR, whereafter the gene was cloned by homopolymer dG/dC tailing. Through cleavage of the terminal BglII sites, the complete VP2 gene with appropriate cohesive termini for cloning into pAcUW3 could be obtained. This would simultaneously eliminate the homopolymer dG/dC tails introduced during cloning, facilitating expression of the foreign gene (Sambrook *et al.* 1989). However, the presence of an internal BglII site necessitated a partial digest of pAHSV3.2, as shown in Fig 3.2. The reaction represented in lane 4 was scaled up to yield sufficient 3.2 kb AHSV-3 segment 2 DNA suitable for ligation into dephosphorylated BglII-restricted pAcUW3. Following transformation of competent Xl1-blue cells, plasmids from a number of the obtained colonies were isolated and characterized by restriction analysis. Recombinant plasmids identified by BglII digestion were subjected to BamHI digestion in order to determine the orientation of the VP2 gene in pAcUW3 relative to the p10 promoter. This was achieved on the basis of a segment 2-specific BamHI site which cuts asymmetrically in the gene, 30 bp from the 5' terminus, and a vector-specific site upstream from the p10 promoter, approximately 400 bp from the BglII insertion site. Thus a plasmid yielding two BamHI fragments of approximately 430 bp and 12900 bp was selected and designated pAcAHSV3.2.

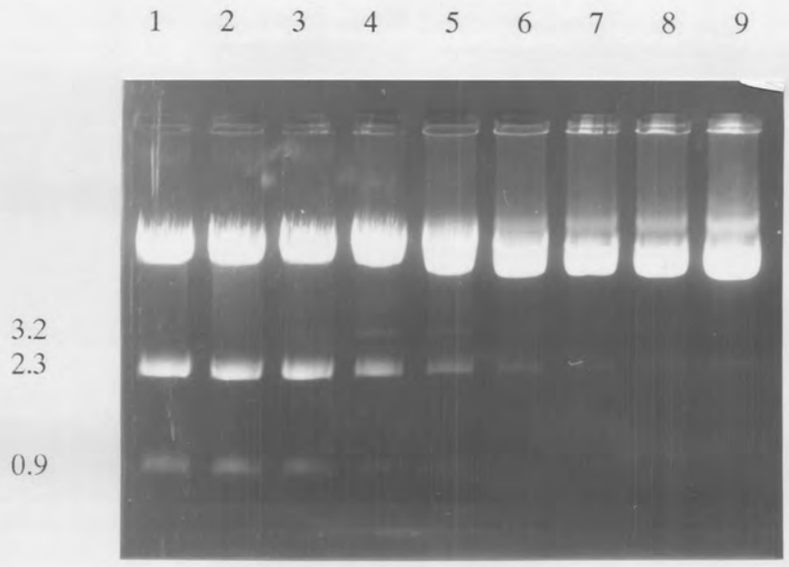

Figure 3.2 An agarose gel electrophoretic analysis of a partial BglII digestion of pAHSV3.2 orchestrated through serial RE dilution. The sizes (in kb) of insert fragments generated through BglII digestion of pAHSV3.2 are indicated, with the full length 3.2 kb AHSV-3 VP2 gene visible in fractions 3 to 6. Overexposure of the photograph was required for clear visualization of the 3.2 kb gene, resulting in poor resolution of the larger fragments.

3.3.2 Production of an AHSV-3 segment 2 baculovirus recombinant

Since the frequency of the homologous recombination event to produce recombinant virus is typically 0.1 to 1%, the identification of a recombinant virus against a background of parental virus can be a very tedious task. Kitts *et al.* (1990) found that engineered derivatives of AcNPV linearized at unique restriction sites had a greatly reduced infectivity, but yielded a higher proportion of recombinants among the progeny viruses from cotransfections with the appropriate transfer vectors, due to the reduced background of wild type viruses rather than an increase in the absolute number of recombinants.

Accordingly, DNA of AcRP23-*lacZ*, a *lacZ* recombinant AcNPV derived by Possee & Howard (1987), was linearized with Bsu36I endonuclease at a unique recognition site within the *lacZ* gene. The recombinant transfer vector pAcAHSV3.2 was CsCl purified, as Sf9 cells are sensitive to some contaminants in crude plasmid preparations and may lyse shortly after transfection (Summers & Smith 1987).

The plasmid and linearised viral DNA were cotransfected into Sf9 cells by lipofection. This method is technically very easy and is routinely used in our laboratory with great success. Samples of the transfected cell supernatants were titrated and screened by staining with Neutral Red containing X-gal. Viruses that retain the *lacZ* gene intact produce plaques that stain blue when incubated with X-gal as opposed to the transparent plaques produced by recombinant viruses. An estimated 25-30% of the plaques produced were transparent. A number of these were picked and screened for VP2 recombinants by hybridization of infected Sf9 cell lysates to a segment 2-specific probe (Fig 3.3). Positive recombinants were subjected to two rounds of plaque purification and used to prepare viral stock solutions of which the titres were determined.

Once the presence of the AHSV-3 segment 2 gene in the baculovirus recombinants had been established, it was necessary to screen for active transcription of the foreign gene by detection of segment 2-specific mRNA in infected cells. This would simultaneously give an indication of the time of maximum expression of VP2 for optimum yields. Sf9 cells were infected with the recombinant baculoviruses and harvested at intervals from 0 to 46 h pi. These cells were spotted onto nitrocellulose, digested with proteinase K and hybridized to a segment 2-specific probe. All recombinants yielded positive signals (results not shown), indicating the active transcription of segment 2 under control of the p10 promoter. The resulting autoradiograph of a selected recombinant, designated AcAHSV3.2, is shown in Fig 3.4. Segment 2-specific RNA was first detected in infected Sf9 cells at 16 h pi (lane 3), followed by stronger positive signals indicating increasing RNA levels to 40 h pi (lanes 4 to 7), whereafter some tapering off occurred toward 46 h pi (lane 8).

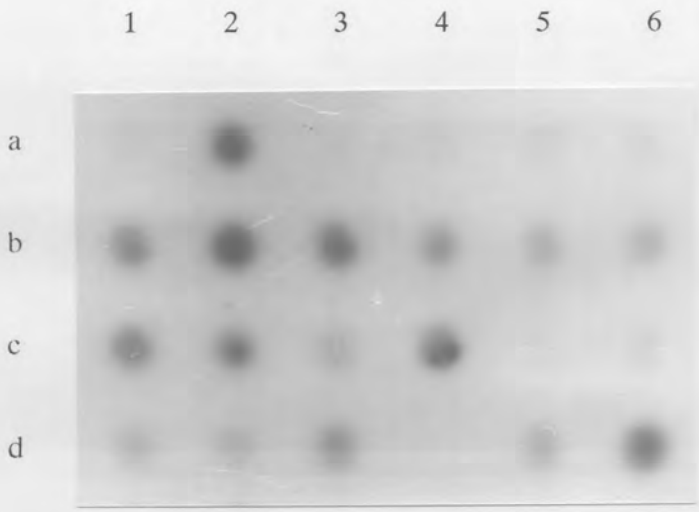

Figure 3.3 An autoradiograph representing plaque hybridization of a ^{32}P-labelled AHSV-3 segment 2-specific insert to lysates of cells infected with potential baculovirus recombinants. Controls were plasmid pAHSV3.2 (a2), wild type AcRP23-*lacZ* infected (a3) and mock infected cells (a4). Dot a1 was marked with bromophenol blue on the membrane for orientation. The baculovirus recombinants represented in b1, b2, b3, c1, c2, c4 and d6 were selected for further analysis.

Figure 3.4 An autoradiograph representing the hybridization of an AHSV-3 segment 2-specific probe with mRNA from mock (a), recombinant baculovirus (b) and wild type AcRP23-*lacZ* (c) infected cell lysates taken at 0 (1), 6 (2), 16 (3), 22 (4), 28 (5), 34 (6), 40 (7) and 46 (8) h pi.

3.3.3 Expression of AHSV-3 VP2 protein in Sf9 cells

In order to determine if AHSV-3 VP2 was synthesized in recombinant virus-infected cells, monolayers of Sf9 cells were infected with recombinant baculoviruses and pulse labelled with ^{35}S-methionine from 28 to 31 h pi. Mock and AHSV-3 infected Vero cells, and mock and wild type AcRP23-*lacZ* baculovirus infected Sf9 cells were similarly prepared as controls. Following harvesting of the cells, the proteins in the total cell lysates were resolved by SDS-PAGE and visualised by autoradiography. The results are shown in Fig 3.5A. A protein of approximately 116 kDa that comigrated with VP2 of AHSV-3 infected cells (lane 2) was shown to be synthesized in recombinant baculovirus infected cells (lane 3), but was found to be absent in mock (lane 5) or wild type AcRP23-*lacZ* infected (lane 4) cells. In the latter case, the 116 kDa ß-galactosidase protein was expressed and migrated as a band of approximately 120 kDa on SDS-PAGE. The additional band in lane 3, corresponding in size to the ß-galactosidase band in lane 4, was identified as a non-specific protein of baculovirus-infected cells through extended electrophoresis, which separated a similar band from the ß-galactosidase band in the wild type AcRP23-*lacZ* infected cells. Confirmation of the viral origin of the putative VP2 protein was obtained by Western immunoblotting of the SDS-PAGE separated proteins (Fig 3.5B). The putative VP2 protein was shown to react specifically with rabbit anti AHSV-3 serum, whereas no interserotype cross-reactivity was observed in Western immunoblots with convalescent horse anti AHSV-4 serum (results not shown). The band of approximately 110 kDa in lane 3 which also reacted with the antiserum may represent a truncated form of VP2. Further analysis of the putative VP2 protein by radioimmuno-precipitation was deemed unnecessary on the basis of the Western blot results.

Recombinant VP2 expressed in infected Sf9 cells was visualized on Coomassie blue stained gels against pulse-labelled AHSV-3 infected Vero cells as marker (Fig 3.6). In order to estimate the amount of VP2 synthesized, lysates of recombinant virus infected cells were electrophoresed along with various known amounts (0.1-3µg) of Rainbow™ marker (Amersham), and visualized by Coomassie blue staining (results not shown). The yield of the expressed VP2 was estimated to be 0.5-1µg/10^6 cells.

On the basis of these results it was concluded that the VP2 protein encoded by genome segment 2 of AHSV-3 was successfully expressed during recombinant baculovirus infection of insect cells.

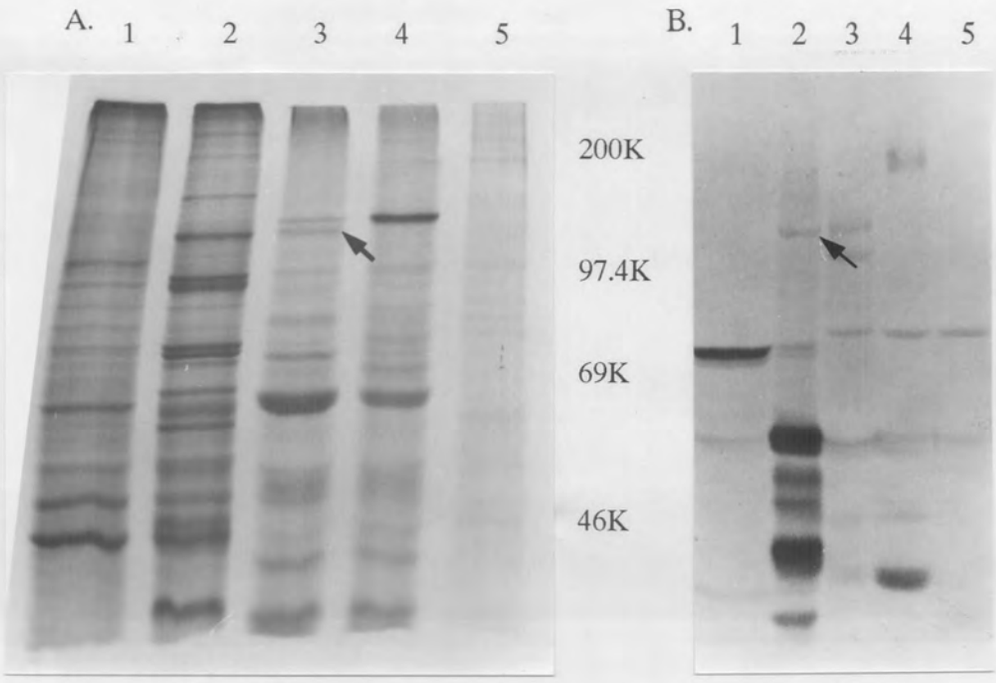

Figure 3.5 Autoradiograph (A) and Western immunoblot (B) of SDS-PAGE separated extracts of mock (1) and AHSV-3 (2) infected Vero cells, AHSV-3 segment 2 recombinant baculovirus (3), wild type AcRP23-*lacZ* (4) and mock (5) infected Sf9 cells. Rabbit anti AHSV-3 serum was used in the Western immunoblot. The arrows indicate the putative recombinant baculovirus expressed VP2 protein.

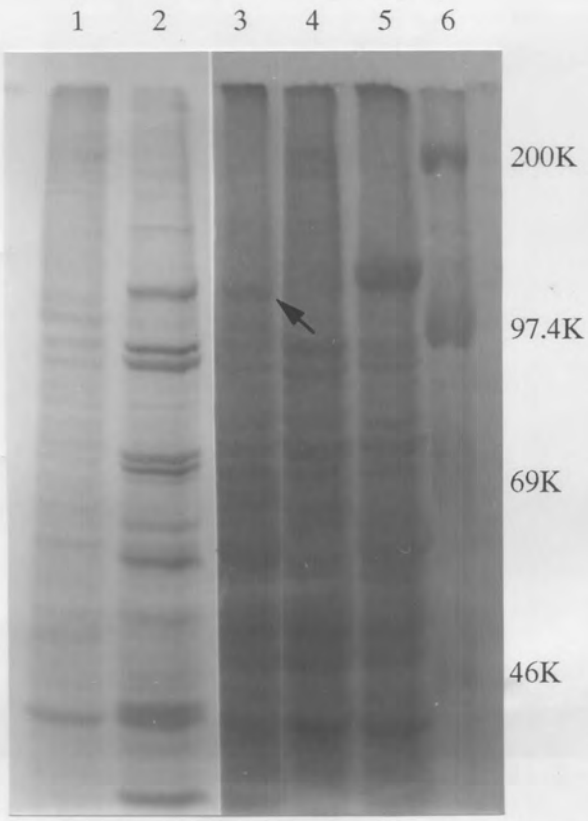

Figure 3.6 SDS-PAGE separation of extracts of mock (1) and AHSV-3 (2) infected Vero cells, AHSV-3 segment 2 recombinant baculovirus (3), mock (4) and wild type AcRP23-*lacZ* (5) infected Sf9 cells. Lanes 1 and 2 were visualized by autoradiography, and lanes 3 to 6 by Coomassie brilliant blue staining. The arrow indicates the recombinant baculovirus expressed AHSV-3 VP2.

3.4 DISCUSSION

In order to evaluate the potential of AHSV VP2 as a subunit vaccine, it is necessary to isolate large quantities of this protein in its native form. In this chapter, the expression of AHSV-3 VP2 in a baculovirus-based eukaryotic expression system suitable for the expression of two proteins, has been described.

Baculovirus expression vectors derived from AcNPV have been widely employed to produce high levels of accurately processed and biologically active proteins (Luckow & Summers 1988; Miller 1988, and references therein). In the case of BTV, cDNA copies of all 10 genome segments have been cloned and expressed in the baculovirus system (reviewed by Roy 1991). The synthesis of individual BTV proteins in cell culture has made a significant contribution to the understanding of BTV morphology as well as the functions of individual proteins. BTV VP2 derived from baculovirus expression vectors has been demonstrated to induce neutralizing antibodies in sheep and to elicit protection against virulent viral challenge (Roy *et al.* 1990), raising the possibility that the expressed protein may be of value as a subunit vaccine for BTV.

As described in the previous chapter, the full length AHSV-3 genome segment 2, which encodes the outer capsid protein VP2, has been cloned by PCR amplification and characterized by sequencing. As noted, minor errors in the sequence of the VP2 gene were detected following the PCR. However, as no shift in the reading frame was expected, it was decided to continue with the expression thereof.

The expression of foreign genes in baculovirus vectors is usually achieved under polyhedrin promoter control (O'Reilly *et al.* 1992). The polyhedrin protein is produced at very high levels in the nuclei of baculoviral infected cells in the late phase of infection and accumulates to form inclusion bodies that contain embedded virus particles. As such, the protein is dispensable for virus replication and only plays a role in the occlusion phase of baculoviral infection. Replacement of the polyhedrin gene with a heterologous gene allows the visual selection of recombinants on the basis of their occlusion negative (occ^-) phenotypes. However, this visual distinction between wild type (occ^+) and recombinant (occ^-) viruses may be difficult without previous experience. An alternative system, utilizing a transfer vector available in our laboratory, pAcDZ1 (Zuidema *et al.* 1990), yields recombinants characterized by the expression of ß-galactosidase, enabling blue/white selection of the recombinants from the wild type viruses in the presence of X-gal. However, ß-galactosidase has the same order molecular weight to AHSV VP2, which would thus complicate the recombinant viral protein analysis. The dual expression transfer vector, pAcUW3, utilizes both the polyhedrin and p10 promoters for the expression of two heterologous foreign genes. The p10 promoter is

responsible for the high level synthesis of a nonstructural protein in wild type baculoviral infected cells (Belyaev & 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 (Williams *et al.* 1989). The p10 promoter has been successfully used to express a number of foreign proteins (Weyer & Possee 1991; Vlak *et al.* 1988; Weyer *et al.* 1990). One of the advantages of the pAcUW3 vector is that, through cotransfection with DNA of a *lacZ* recombinant baculovirus, AcRP23-*lacZ* (Possee & Howard 1987), recombinants can be selected by blue/white selection. Of greater importance, however, in the search for an efficacious subunit AHSV vaccine and the construction of VP2 baculovirus recombinants, is the possible future application of the vector in multiple expression of the AHSV capsid proteins.

The tailored DNA copy of AHSV-3 genome segment 2 was cloned into pAcUW3 under p10 promoter control. Following cotransfection into Sf9 cells with linearized AcRP23-*lacZ* DNA, the high proportion (approximately 25%) of recombinant baculoviruses obtained suggested that linearization of the baculoviral DNA prior to cotransfection with the transfer vector was successful. It should be noted, however, that this is due to the reduced background of wild type virus rather than an increase in the absolute number of recombinants.

The presence of AHSV-3 segment 2-specific DNA in potential recombinant baculovirus infected cells was confirmed and was found to yield maximal levels of mRNA at 40 h pi, in accordance with transcription from the very late p10 promoter. AHSV-3 VP2 with a similar electrophoretic mobility to VP2 of AHSV-3 infected cells, was shown to be synthesized in recombinant baculovirus-infected cells by radiolabelling and Western immunoblotting, albeit at low levels (0.5-1$\mu g/10^6$ cells) as adjudged in Coomassie blue stained gels.

As the factors which determine how well a foreign gene is expressed in the baculovirus system are not well characterized, it is difficult to predict how efficiently different genes will be expressed (Summers & Smith 1987). The level of AHSV-3 VP2 expression (0.5-1$\mu g/10^6$ cells) is slightly lower than the reported baculovirus expression levels of BTV-10 VP2 (1-3$\mu g/10^6$ cells; Inumaru & Roy 1987) and AHSV-4 VP2 (2$\mu g/10^6$ cells; Martinez-Torrecuadrada *et al.* 1994). Varying levels of baculovirus expression of other neutralizing antibody-eliciting viral proteins have been reported, including rabies virus glycoprotein (12$\mu g/10^6$ cells; Prehaud *et al.* 1989), rift valley fever virus envelope glycoprotein (2-10$\mu g/10^6$ cells; Schmaljohn *et al.* 1989), Mokola virus glycoprotein (3.2$\mu g/10^6$ cells; Tordo *et al.* 1993) and BHV-1 glycoprotein IV (15-35$\mu g/10^6$ cells; van Drunen Littel-van den Hurk *et al.* 1993).

Matsuura *et al.* (1987) have studied the requirements for optimal expression from the polyhedrin promoter in order to understand the reasons for the reduced levels of synthesis of

foreign gene products as opposed to the level of the wild type protein. All the available data indicate that the level of expression of a foreign gene that replaces the AcNPV polyhedrin gene is related to the integrity of the immediate 5' upstream sequences of the polyhedrin gene. We have utilized the BglII site in the pAcUW3 dual transfer vector for expression under p10 promoter control. pAcUW3 contains the complete p10 5' leader sequence and the first nucleotide of the p10 translation initiation codon, which is very similar to the vector, pAcYM1, which was used by Matsuura *et al.* (1987) to obtain optimal expression from the polyhedrin promoter. In the latter case, however, it was noted that the flanking sequences of the initiatiating ATG translation codons of the polyhedrin and foreign genes were similar. It was unknown whether this had any bearing on the level of translation of the respective gene products. In this study, the 12 bp 5' noncoding region of the AHSV-3 VP2 gene was left intact in the construction of the recombinant baculovirus. It was not investigated whether modification or shortening of this region may have had any effect on the level of AHSV VP2 expression. This may account for the slightly lower expression levels obtained compared to those of AHSV-4 VP2 obtained by Martinez-Torrecuadrada *et al.* (1994), where the ORF of the VP2 gene was flanked at its 5' end only by three A's and the restriction site used for cloning.

A number of studies on the control of reovirus mRNA translation efficiency have been undertaken. Although the 10 species of reovirus mRNA are synthesized in roughly equimolar amounts in infected cells, they are translated with frequencies that differ by as much as 100-fold (Levin & Samuel 1980; Gaillard & Joklik 1985). It was found that the differential translation of the S1 and S4 mRNAs *in vivo* could be attributed to intrinsic structural properties of the individual mRNAs, and was independent of competition of other viral mRNAs (Atwater *et al.* 1987; Samuel & Brody 1990). The 5' region upstream of the initiation codons of different mRNAs also directly influenced their translation efficiency. This included not only the Kozak sequence, but also the length and the secondary structure of this whole 5' region (Roner *et al.* 1989). Thus the factors governing the translation efficiency of a specific protein are multiple and complex and could be intrinsic to the protein.

One possible explanation for the low levels of detectable AHSV VP2 in infected cells was supplied by an experiment performed in the Department of Genetics at the University of Pretoria (Van Staden *et al.* in press, unpublished results), which indicated a toxic effect of VP2 on Sf9 cells, a hypothesis echoed by Martinez-Torrecuadrada *et al.* (1994) concerning the baculovirus expression of AHSV-4 VP2. Sf9 cells were infected with baculovirus recombinants expressing the AHSV-3 structural protein VP2 (as described here), or the non-structural proteins NS2 or NS3. The latter recombinants, which were constructed within the department, yielded 50µg NS2 or 0.1µg NS3/10^6 Sf9 cells under baculovirus polyhedrin promoter control.

Time curves of the percentage of viable cells, as ascertained by impermeability to trypan blue staining, indicated that the viability of cells infected with VP2-expressing baculovirus recombinants was reduced significantly more than with NS2 recombinants, but less than with NS3 recombinants. These observations were also reflected in time curves of the AHSV-specific mRNA levels in the infected cells. As NS2 is packaged in virus-inclusion bodies (VIBs) in infected cells, it can be postulated that NS2 is efficiently removed from the cytoplasm and stored in a 'harmless' form, whereas no similar system is operating for VP2 and NS3. However, reasons for the observed effects were not further investigated.

Although AHSV-3 VP2 has a predicted molecular weight of 123 kDa based on the amino acid sequence data, both the wild type viral protein expressed in Vero cells and the recombinant baculovirus-expressed protein migrate as proteins of approximately 116 kDa, estimated from protein standards with known molecular weights. Grubman & Lewis (1992) similarly estimated the molecular weight of AHSV-4 VP2 as 107 kDa, although the calculated value from amino acid sequence data was 124 kDa (Iwata *et al.* 1992). ß-galactosidase, expressed in the wild type AcRP23-*lacZ* baculovirus-infected Sf9 cells, migrated as a protein of approximately 120 kDa, while its predicted molecular weight is 116 kDa. These discrepancies in the calculation and SDS-PAGE resolution of molecular weights may be related to post translational modifications undergone by the protein, or to inaccuracies in the extrapolation from protein standards with non-equivalent molecular weights.

The recombinant baculovirus-expressed AHSV-3 VP2 was shown to exhibit no interserotype cross-reactivity with AHSV-4 antiserum in Western immunoblotting, confirming the serotype-specificity of VP2. In subsequent studies, not included in this thesis, the ability of baculovirus expressed AHSV-3 VP2 to induce a neutralization-specific immune response was assessed. Plaque reduction assays were conducted with antibodies raised in rabbits and guinea pigs to lysates of recombinant baculovirus-infected Sf9 cells, demonstrating low but detectable titres in 3 of the 4 animals injected. These results confirmed the findings of Burrage *et al.* (1993) that AHSV VP2 carries the neutralization-specific epitopes and support the potential of VP2 as a possible subunit vaccine to AHSV.

The baculovirus system is continually being improved, and one of the latest innovations is the construction of a novel baculovirus shuttle vector (bacmid) that can replicate in *E. coli* as a plasmid, and can also infect susceptible insect cells (Luckow *et al.* 1993). Using this system, the time required for producing and purifying a recombinant virus is reduced from the conventional 4 to 6 weeks to only 7 to 10 days, partly because the recombinant virus DNA is produced and selected in *E. coli*, and not in insect cells. This could greatly facilitate attempts at improving the expression levels of AHSV VP2, as well as the expression and screening of other antigenic AHSV proteins.

CHAPTER 4

CONCLUDING REMARKS

In the proceedings of the second international symposium on bluetongue, African horsesickness and related orbiviruses, B.I. Osburn (1992) reported that studies regarding immune responses to orbiviruses have, with exceptions, identified VP2 as the primary, and possibly the only viral protein responsible for inducing virus neutralizing antibody.

The objective of this study was to investigate VP2 of AHSV-3 as the main determinant of serotype-specificity and the neutralization-specific immune response. In particular, the envisaged approach to this investigation involved the cloning and characterization, through sequencing, of the gene that encodes VP2, and the expression of the protein in a eukaryotic expression system. The results have been discussed in detail in the relevant preceding chapters. In this chapter, the pertinent information which contribute to a better understanding of the molecular biology of AHSV will be mentioned briefly, and some suggestions for future research in this field will be made.

The nucleotide sequence of genome segment 2 dsRNA was determined and represents only the second AHSV VP2 gene sequence published. This enabled the first analysis of the inter serotype variation of the AHSV VP2 gene and protein. The variability exposed by these comparisons fell within the range of the inter BTV serotype variability associated with the gene encoding the main determinant of serotype-specificity. Further VP2 sequences would be required to speculate on the evolutionary divergence of the different AHSV serotypes. The orbiviral serogroups could be clearly distinguished by VP2 sequence comparisons. AHSV was found to be more distantly related to BTV than EHDV. Nonetheless, a common ancestry of the AHS viruses and other orbiviruses was revealed through the identification of AHSV- and orbivirus-specific sequence conservation.

It was shown that AHSV genome segment 2 exhibits the conserved terminal hexanucleotides, with a segment-specific inverted terminal repeat, reported for other orbivirus segments. Translation of the ORF nucleotide sequences yielded VP2 proteins variable in amino acid sequence, but similar in structural characteristics. Two highly variable areas were identified, one displaying significant hydrophilicity, and it was postulated that this could represent a serotype-specific epitope of AHSV VP2. The COOH-terminal was found to be relatively conserved, as is also the case with BTV VP2. This conservation could be indicative of a role in the maintenance of the three-dimensional structure of the protein, although no postulated explanations have yet been investigated.

The strategy described for the cloning of the AHSV-3 VP2 gene in this study has already been succesfully applied to the cloning of other AHSV genes in the laboratory, including the other capsid genes. This paves the way for further investigation and characterization of all the genes of AHSV.

A further objective of this investigation was to express the neutralization-specific antigen of AHSV in a eukaryotic system, which has been achieved in the case of AHSV-3 VP2 through baculovirus expression. The expressed protein was shown to be reactive with anti AHSV-3 serum, but not with anti AHSV-4 serum, supporting the probability of VP2 as the serotype-specific antigen.

Although the level of expression of VP2 in this study turned out to be relatively low, the ability to express AHSV VP2 in a eukaryotic expression system paves the way for further study into the immunogenicity of VP2, particularly with respect to the capabilities of eliciting a neutralization-specific immune response. In addition, the characterization of the neutralizing epitopes and the interaction of the structural proteins in virus neutralization require further investigation. In particular, studies evaluating the contribution of VP5 and other structural proteins to AHSV neutralization will be necessary to determine their role in protective immunity and should be taken into account in the context of the production of efficacious vaccines. Coexpression of VP2 with other capsid proteins and the assembly of VLPs and CLPs would present a number of opportunities for further investigation, including the stages of viral assembly, the contributions of individual components to that process, and the sites and nature of viral protein interactions. An increased understanding of viral morphogenesis may aid the development of antiviral agents which specifically interfere with the assembly process. Intact VLPs could also prove useful as vaccines if epitopes are presented in authentic conformations.

On the vaccine development front, additional research must address the gaps in our understanding of the basis for protective immunity. The contributions of specific immune processes in modulating the course of primary and recurrent AHSV infections is essential to the construction of efficacious vaccines which will effectively mimic natural AHSV infection, yet provide superior protection against primary infection and the establishment of latency. Studies directed at associating specific immune responses, cellular versus humoral, with protective immunity are for the most part inconclusive and often contradictory. Characterization of the cellular immune responses to orbivirus infections, specifically cytotoxic T-lymphocytes, have been limited to demonstration of their existence and permissive specificity to BTV serotype. Thus, while it is apparent that both antibody- and cell-mediated reactions are effective defense mechanisms which may act in concert to clear infections, the exact *in vivo* roles of these processes have not yet been fully elucidated, and little information on their protein or peptide specificities has been reported. The cloning and expression of the individual

proteins of AHSV represents a possible gateway to the investigation of these reactions.

In addition, antigenic competition, selection of appropriate serotypes for global and regional needs, technical complexity of the manufacturing process, volume limitations, affordability, safety and efficacy also remain important issues in vaccine production.

In conclusion, it may be stated that the VP2 gene of AHSV-3 has been cloned, sequenced and expressed, forming an excellent basis for the ongoing research into the development of a recombinant vaccine for AHSV, with potentially important academic and economic implications.

Parts of the results presented in this thesis have been published:

Vreede, F.T. & Huismans, H. 1994. Cloning, characterization and expression of the gene that encodes the major neutralization-specific antigen of African horsesickness virus serotype 3. *J. Gen. Virol* 75, 3629-3633.

Parts of the results presented in this thesis have been presented at scientific meetings:

Huismans, H., Van Schalkwyk, T, Vreede, F.T. & Bremer, C.W. Cloning and characterization of the African horse sickness genes that encode the protective outer capsid antigens. Twelfth Congress of the South African Genetics Society, Johannesburg, 1990.

Vreede, F.T., Durbach, S., Cormack, S.B. & Huismans, H. The cloning and characterization of the gene of African horse sickness virus which could be used in the development of a recombinant vaccine. First South African Biotechnology Conference, Grahamstown, 1993.

Vreede, F.T. & Huismans, H. Cloning, characterization and expression of the VP2 gene of African horse sickness type 3. Ninth International Congress of Virology, Glasgow, 1993.

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