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Characterisation and co-expression of the two outer capsid proteins of African horsesickness virus serotype 3

by

Renate Dorothea Filter

Submitted in fulfillment of the requirements for the degree Magister Agriculturae Scientiae to the in the Faculty of Agricultural and Biological Sciences (Department of Genetics) University of Pretoria, Pretoria

January 2000

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I declare the thesis, which I hereby submit for the degree Magister Agriculturae Scientiae at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at another university.

R Filter
Renate Dorothea Filter

16/1/2000
Date

Difficult things take a long time, the impossible takes a little longer.

Chaim Weizmann

(Israeli Chemist)

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SUMMARY

Characterisation and co-expression of the two outer capsid proteins of African horsesickness virus serotype 3

by

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Promoter: Prof. H. Huismans
Department of Genetics
University of Pretoria

For the Degree MSc(Agric)

African horsesickness is caused by the AHSV, a member of the genus *Orbivirus*, family *Reoviridae*. Nine serotypes have been identified. The viral genome consists of ten double stranded (ds) RNA segments encoding at least 7 structural and 4 non-structural proteins. The major core proteins VP3 and VP7 together with the minor core proteins VP1, VP4 and VP6 form the core particle surrounding the 10 dsRNA segments. An outer capsid, consisting of two major structural proteins VP2 and VP5 surrounds the core. VP2 is the most variable of the proteins within the AHSV serogroup and carries serotype specific epitopes which induce a protective immune response against virulent homologous AHSV challenge. The VP2 protein is therefore the antigen of choice for the development of a subunit vaccine against AHSV. It has been shown that protection against AHSV-4 can be achieved by vaccination with AHSV VP2 protein.

The AHSV-3 VP2 protein has previously been cloned and expressed as baculovirus recombinant protein in our laboratory. The recombinant protein induced only a weak neutralising immune response. It has been determined in this investigation that the majority of recombinant AHSV-3 VP2 proteins expressed in Sf-9

insect cells are in an insoluble, aggregated form. This is likely to be the cause of the poor neutralising immune response induced by this protein.

In order to investigate this problem two strategies were adopted. First an attempt was made to chemically solubilise the particulate VP2 protein and refold the protein into a form that may present the neutralising epitopes more appropriately. The solublisation of the protein with 6M Guanidinium HCl was successful, but the largest percentage of the protein was again rendered insoluble during the refolding process which involves the removal of Guanidinium HCl by column chromatography. The chemical solubilisation therefore proved to be too inefficient to provide a solution to the problem.

The second method for increasing the solubility and immunogenicity of the VP2 protein was by co-expression of VP2 and VP5, the two outer capsid proteins of AHSV-3. For the dual expression of the two proteins it was necessary to characterise the AHSV-3 VP5 gene and express it as a baculovirus recombinant first. The VP5 gene was therefore sequenced. A nucleotide sequence of 1566 bp was determined encoding a peptide of 505 amino acids with a predicted size of 56K. The VP5 was expressed as baculovirus recombinant using the baculovirus Bac-to-Bac™ expression system. The yield of VP5 was low but was nevertheless better than the expression levels of AHSV-9 VP5 gene using an alternative baculovirus expression system.

AHSV-3 VP2 and VP5, were cloned respectively under the polyhedrin and p10 promoters of the pFastbac dual transfer vector of the Bac-to-Bac™ baculovirus expression system. mRNA transcription of both AHSV-3 VP2 and VP5 genes in Sf-9 cells was shown. The expression of VP2 was also demonstrated but VP5 was very poorly expressed by the dual recombinant. Further research to determine the effect co-expression of AHSV-3 VP5 in the AHSV-3 VP2 antigenicity is needed.

OPSOMMING

Karakterisering en ko-ekspressie van die 2 buite dop proteïne van Perdesiekte virus serotipe 3.

deur

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Vir die graad MSc(Agric)

Perdesiekte word veroorsaak deur die PSV, 'n lid van die *orbivirus* genus in die familie *Reoviridae*. Nege serotipes is identifiseer. Die virus genoom bestaan uit 10 ddRNA segmente wat vir ten minste 7 strukturele and 4 nie-strukturele proteïne kodeer. VP3 en VP7 is die hoof kern proteïne wat saam met die mindere kern proteïne, VP1, VP4 en VP6 die kern partiekel vorm wat die ddRNA genoom omsluit. Die buite dop bestaan uit die hoof dop proteïne VP2 en VP5 wat die kern omsluit. VP2 is die mees variabele proteïen binne die PSV serogroep en dra die serotiepe spesefieke epitope wan 'n beskermende imuun respons induseer teen homoloë PSV infeksie. VP2 is dus die verkiesde kandidaat vir die ontwikkeling van 'n sub-eenheid vaksien teen PSV. Beskerming teen PSV-4 is al verkry met rekombinante PSV-4 VP2.

Die PSV-3 VP2 proteïen is voorheen in ons laboratorium gekloneer, en uitgedruk as baculovirus rekombinante proteïen. Ongelukkig is daar geen meetbare neutraliserende teenliggame deur die rekombinant PSV-3 VP2 uitgelok nie. Ons het vasgestel dat die mederheid van die rekombinate PSV-3 VP2 wat in insek selle uitgedruk word in 'n partikulêre vorm voorkom. Dit mag die rede wees vir die swak neutraliserende immunrespons teen hierdie proteïen.

Twee strategieë is gevolg om hierdie probleem op te los. 'n Poging is aangewend om met chemiese oplosmiddels die partikulêre PSV-3 VP2 op te los en daarna te hervou in 'n vorm wat neutraliserende epitope meer effektief vertoon. 6M guanidien hidrochloried is suksessvol gebruik om die partikulêre VP2 op te los, maar 'n groot hoeveelheid van die proteïen het weer geaggregeer deur die hervouings proses. Chemiese oplossing en hervouing was moontlik, maar te ondoeltreffend om 'n oplossing vir die probleem te bied

Om die oplosbaarheid en immunogenisiteit van die VP2 proteïen te verbeter is die gelyktydige uitdrukking van die VP2 en VP5 proteïne van PSV-3 ondersoek. Vir die gelyktydige uitdrukking van die twee proteïne was dit nodig om PSV-3 VP5 geen the karakteriseer en as baculovirus rekombinant uit te druk. Die nukleotied volgorde van die PSV-3 VP5 geen is 1566bp lank en kodeer vir 'n pepdied van 505 aminosuure met 'n geskatte groote van 56K. Die PSV-3 VP5 is as baculovirus rekombinant uitgedruk met behulp van die Bac-to-Bac™ uitdrukkings sisteem. Uitdrukkings vlakke was laag, maar beter as die van PSV-9 VP5.

Die PSV-3 VP2 is onder die polyhedrin promoter en VP5 onder die p10 promoter van die pFastbac Dual oordrag vektor van die Bac-to-Bac™ gekloneer. Vir beide VP2 en VP5 van PSV-3 kon transkripsie van mRNA aangedui word. VP2 sintese kon ook aangedui word. VP5 uitdrukking met die pFastbac Dual sisteem was egter baie swak. Verdere navorsing is egter nodig om die effek van PSV-3 VP5 op PSV-3 VP2 se antigenisiteit te bepaal

Abbreviations and symbols

[ ]	concentration
α	alpha
β	beta
λ	lambda
μ	mu
θ	theta
σ	sigma
μCi	microcurie
μg	microgram
μl	microlitre
A	adenosine
AHS	African Horsesickness
AHSV	African Horsesickness virus
AHSV-3	African Horsesickness virus serotype 3
Amp	ampere
ATCC	American type culture collection
bp	base pairs / basis pare
BSA	bovine serum albumin
BT	Bluetongue
BTV	Bluetongue virus
C	cytosine
°C	degrees Celsius
cDNA	complementary DNA
CER	Chicken erythrocyte cells
Ci	Curie
CLP	core like particle
CTL	cytotoxic T lymphocyte
cm	centimetre
cs	cell surface

dATP	2'-deoxyadenosine-5'-triphsphate
dCTP	2'-deoxycytoine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
DMF	dimethylformamide
DMSO	dimethylsulfoxide
dNTP	2'-deoxynucleoside-5'-triphosphate
ddNTP	2',3'-deoxynucleoside-5'-triphosphate
DNA	deoxyribonucleic acid
ds	double stranded
dd	dubbeldraad
DTT	1,4,-dithiotreitol
E.coli	*Escherichia coli*
EDTA	ethylenediaminetetra-acetic acid
EHDV	Epizootic haemorrhagic disease virus
et al	*et alia* (and others)
EtBr	Ethidium bromide
FCS	fetal calf serum
Fig.	Figure
G	guanidine
g	gram
GHCl	guanidine hydrochloride
GTP	guanosine triphosphate
h	hour
hpi	hours post infection
IgA	Immunoglobulin A
IPTG	isopropyl-β-D-thiogalactopyranoside
ISVP	infectious subunit core vriuses
K	kilodalton
l	litre

LB	Luira –Bertani
log	logarithmic
M	Molar
Mab	monoclonal antibody
mAmp	milliampere
mCi	millicurie
MES	2-[Morpholino] ethane sulfonic acid
mg	milligram
min	minute
ml	millilitre
mM	millimolar
mm	millimetre
mmol	millimole
MMOH	methylmecuric hydroxide
m.o.i	multiplicity of infection
MOPS	3-[N-morpholino] propane sulfonic acid
NaAc	sodium acatate
ng	nanogram
nm	nanometre
NS	non-structural
OD_{550}	optical density at 550nm
OVI	Onderstpoort Veterinary Institute
PAGE	polyacrylamide gel electrophoresis
PCR	polymarase chain reaction
PEG	poly-etheleneglycol
pi	post infection
pfu	plaque forming units
PGL	poly (lactide-coglycolide)
PSV	Perdesiekte virus
RE	restriction endonuclease

RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
s	second
SDS	sodium dodecyl sulphate
Sf-9	*Spodoptera frugiperda*
ss	single stranded
T	thymidine
TEMED	N,N,N',N',-tetramethylethylenediamine
Tris	Tris(hydroxymethyl)-aminomethane
Tris HCl	Tris(hydroxymethyl)-aminomethane hydrochloride
U	units
U	uridine
UV	ultraviolet
V	volt
v	volume
VIB	virus inclusion body
VLP	virus like particle
v/v	volume per volume
VP	virus protein
w	weight
w/v	weight per volume
w/w	weight per weight
X-gal	5-bromo-4-chloro-3-indonyl-β-D-galactopyranoside

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CHAPTER 1

Literature Study

1.1 Introduction

Regular epidemics related to African Horsesickness (AHS) have been recorded over the centuries. The first of which was a report in an Arabian document in year 728 (Henning, 1956). In South Africa, since the colonisation of the Cape, epidemics were recorded approximately every 30 to 40 years (Coetzer & Erasmus, 1994, Henning, 1956). In 1854, 70 000 horses representing 40% of the horse population in the Cape of Good Hope died of African horsesickness (Henning, 1956). In 1987-1990, an African horsesickness outbreak was recorded in Spain resulting in the loss of hundreds of horses (Rodriguez *et al.*, 1992). More recently in 1996, an AHS outbreak occurred in South Africa. This outbreak resulted in at least 500 recorded fatalities. Vaccination against AHSV prevented a greater loss (Meiswinkel, 1998). The prevention of African horsesickness related epidemics is therefore important. An epidemic can result in both stock and financial loss with severe effects on the modern horse industry. The epidemiology of the AHS and the characterisation of its aetiological agent are of fundamental importance in the attempts to manage the disease.

African horsesickness is a non-contagious, infectious, often fatal, vector borne disease of equines (House, 1993a, Mellor, 1993) and sometimes dogs (Alexander *et al.*, 1995). The disease is endemic to Sub-Saharan Africa (Mellor, 1994, Mellor & Boorman, 1995). However outbreaks have also been recorded in the Middle East (Coetzer & Erasmus, 1994), Morocco, Spain and Portugal (House, 1993a, Mellor, 1994, Mellor & Boorman, 1995). The aetiological agent, African horsesickness virus (AHSV) (McIntosh, 1958), is transmitted by biting arthropod of the *Culicoides* species (Du Toit, 1944, Mellor, 1993, Mellor, 1994). AHSV is a member of the genus Orbivirus, family *Reoviridae* (Gould & Hyatt, 1994, Oellermann *et al.*, 1970). Nine AHSV serotypes have been identified (Gould & Hyatt, 1994, McIntosh, 1958). The virus consists of a segmented dsRNA genome enclosed by a double capsid shell. The

genome encodes at least 10 virus proteins. The inner shell consists of 3 minor proteins; VP1, VP4 and VP6 surrounded by two major core proteins VP3 and VP7. The outer capsid consists of two major outer capsid proteins, VP2 and VP5. VP2 has been shown to carry neutralising serotype specific epitopes. The VP2 protein is therefore a candidate for the development of subunit vaccine, a possible alternative to the available live attenuated virus vaccine used presently against AHSV infection (Roy *et al.*, 1994b).

To manage AHS and prevent possible epidemics the epidemiology, pathogenicity and molecular biology of the AHSV have to be fully understood. This knowledge will aid in the future management of the disease and prevention of AHS and related diseases.

1.2 Classification, epidemiology and pathogenesis of AHSV

1.2.1 Classification:

AHSV is an orbivirus, one of the eight genera in the *Reoviridae* family of dsRNA viruses. The prototype member of the orbiviruses is bluetongue virus (BTV). Other well-known members of the *Reoviridae* family include the rotaviruses and reoviruses (Nibert, 1998).

Members of the *Reoviridae* family are highly diverged with regard to host, transmission, geographic localisation and pathological consequences (Urbano & Urbano, 1994) but similarities between the viruses can be defined. These similarities however are tentative. Only cautious comparisons between the protein functions and structures of the members of the *Reoviridae* can be made (Nibert, 1998). The viruses lack lipid envelopes and are isometric in form. A 10-12 dsRNA segmented genome is protected by an inner core surrounded by one or two distinctive icosahedral capsids (Nibert, 1998). Virus replication takes place in the cell cytoplasm where large inclusions develop containing dsRNA and structural proteins. Parachrystaline arrays of progeny virions develop in the inclusions (Urbano & Urbano, 1994). Proteins of

specific activities have been found to be present in these viruses. Functions of cell binding and penetration, RNA polymerases, RNA guanylyl transferases and others have been identified in most studied *Reoviridae* members (Nibert, 1998).

The orbiviruses are a clearly defined, but a particularly large and diverse group within the *Reoviridae* family, with similar morphological and physiochemical properties (Gorman & Taylor, 1985, Urbano & Urbano, 1994). The orbiviruses are distinguishable from other *Reoviridae* members by the fact that they infect both insects and vertebrates and that the infectivity of the virus is lost in mild acid conditions. The orbiviruses, in contrast to reoviruses, have an outer shell with no distinctive capsomeric structure. The inner shell has a distinctive structure consisting of the 32 ring-shaped capsomeres arranged in icosahedral symmetry. Hence the name orbivirus from the Latin '*orbis*' meaning ring. There are twelve serological groups of orbiviruses. Each group consists of several serotypes (Table 1.1) (Gorman & Taylor, 1985, Gould & Hyatt, 1994). Bluetongue virus (BTV), the orbivirus prototype, consists of 24 serotypes and has been extensively studied. AHSV, with 9 serotypes, has many similarities with BTV but also some differences have been identified (Gorman & Taylor, 1985, Gould & Hyatt, 1994).

Table 1.1: Orbivirus serological groups (Gould & Hyatt, 1994)

Serogroup	Number of serotypes
African Horsesickness	9
Bluetongue	24
Changuinola	5
Corriparta	4
Epizootic haemorrhagic disease virus	8
Equine encephelosis virus	5
Eubenangee	3
Kemerovo	21
Palyam	6
Wallal	2
Warrego	2
Ungrouped	

1.2.2. Epidemiology and Pathogenesis

African horsesickness is endemic to Sub-Saharan Africa occurring naturally in zebras (Lord *et al.*, 1997), elephant and buffalo (Coetzer & Erasmus, 1994). All nine AHSV serotypes occur in South Africa. However, outbreaks have also been recorded in Mediterranean countries, North Africa and the Middle East. Epidemics often occur in the warm, wet summer months. This weather is ideal for proliferation of the AHSV vectors, biting midges of the *Culicoides* species, therefore spreading the virus. (Coetzer & Erasmus, 1994, Du Toit, 1944, Meiswinkel, 1998, Mellor, 1994).

The virus causes disease in horses, donkeys, mules and sometimes in dogs (Alexander *et al.*, 1995, Coetzer & Erasmus, 1994, Mellor, 1994). The mortality rate of AHS in horses can be up to 95%. This depends on the virulence of the virus, the immune status and the type of infected horse (Coetzer & Erasmus, 1994).

The clinical signs of AHSV infections in horses vary greatly. They are classified into three categories: The 'Dunkop' or pulmonary, 'Dikkop' or cardiac and the mixed form with symptoms of both pulmonary and cardiac forms. The pulmonary form is the most severe with a 95% mortality rate. The clinical signs are fever, coughing and large quantities of serofibrinous fluid discharge from the nostrils. The cardiac form is less severe and results in swelling of the neck and head particularly the supraorbital fossae. Fifty percent of animals infected with the cardiac form survive. The mixed form is the most common form of AHS and presents itself clinically in a combination of the pulmonary and cardiac form resulting in a mortality rate of 70% (Coetzer & Erasmus, 1994).

Vaccination programmes are implemented to control possible AHS epidemics. Live attenuated virus vaccines are produced by repetitive passage in mouse brains, eggs or monkey kidney cells (House, 1998). Currently immunisation in endemic regions of AHSV consists of two consecutive courses of quadrivalent vaccines containing attenuated strains of serotypes 1, 3, 4, and 5 followed by serotypes 2, 6, 7 and 8 (Coetzer & Erasmus, 1994). This vaccine protects against all serotypes because

of the low, but significant level of crossprotection of specific serotypes (House, 1998). It was suggested that the use of the live attenuated polyvalent vaccine reduced the potential epidemic proportions of the 1996 AHS outbreak in South Africa (Meiswinkel, 1998). The current AHSV vaccine is highly effective but drawbacks of attenuated live vaccines are relevant. Ineffective attenuation, reversion of attenuated viruses to virulence, recombination with wild type viruses and difficulty in distinguishing between vaccinated and infected horses are current concerns. Live attenuated vaccines also cannot be used in non-endemic areas and therefore an AHSV-4 specific inactivated vaccine was developed for countries such as Spain (House, 1993b, House, 1998). Alternative new generation vaccines against AHSV are currently under investigation to overcome the drawbacks of live attenuated vaccine.

The different approaches for new generation vaccines against AHSV have to be carefully investigated and evaluated to develop a vaccine against AHSV that is safe, effective and affordable (House, 1993b). To achieve this the molecular biology of AHSV has to be clearly understood.

1.3 Molecular biology of AHSV

The molecular biology of bluetongue (BTV), the orbivirus prototype, has been extensively studied. Much less is known about the AHSV. Since the two viruses are very similar in many respects the following review will concentrate on known factors about the AHSV. Where possible and relevant analogies to BTV and other *Reoviridae,* such as rotavirus and reovirus, will be drawn to elucidate some aspects or highlight important differences.

1.3.1 AHS virion

The AHS virion consists of 10 dsRNA segments (Figure 1.1) protected by a double capsid of 7 structural proteins . The virus core consists of three minor core proteins; VP1, VP4 and VP6 surrounded by two major core proteins VP3 and VP7. The

genome is found within the core particle of the virus. The outer capsid of the virus consists of VP2 and VP5, the major capsid proteins (Figure 1.2)(Roy & Sutton, 1998).

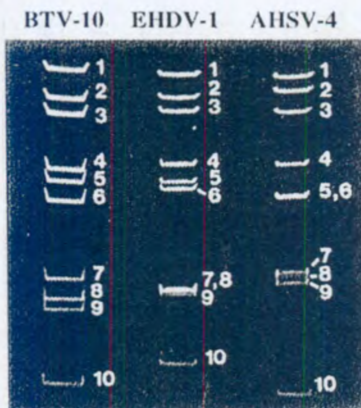

Figure 1.1: SDS Page gel of BTV, EHDV and AHSV dsRNA genomes (Roy & Sutton, 1998).

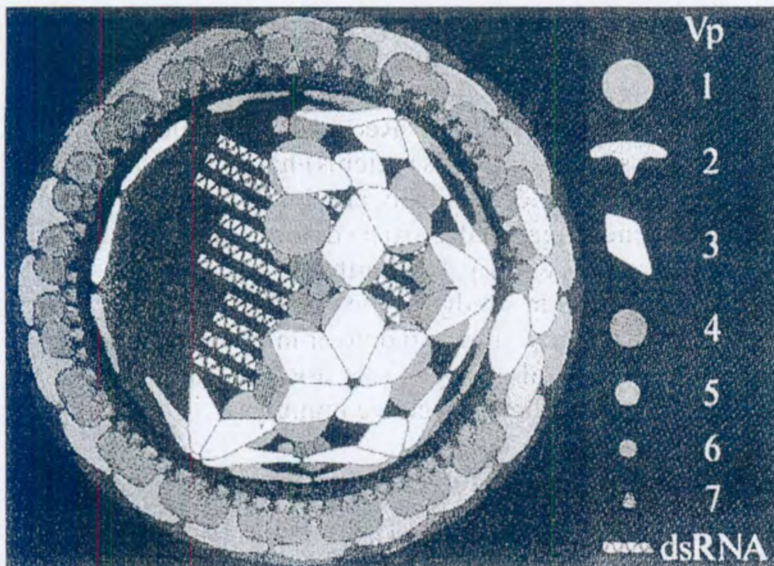

Figure 1.2: BTV structure (Roy & Sutton, 1998)

The complete virion is infective in mammalian and *Culicoides* cells. The infectivity of AHS infectious subunit virus particles (ISVP) does not differ significantly from that of AHS virions in mammalian cells (Burroughs *et al.*, 1994, Marchi *et al.*, 1995, Roy *et al.*, 1994b). The ISVP's can be generated artificially by the cleavage of VP2 with chymotripsin (Xu *et al.*, 1997). Core particles however, produced by the removal of VP2 and VP5, are 10^5 fold less infectivity in mammalian cells (Burroughs *et al.*, 1994). In *Culicoides* cells however the ISVP's seem to be more infective than AHSV virions. Preferential binding of core particles of BTV to *Culicoides* cells (Xu *et al.*, 1997) supports indications that AHS ISVP's may be more infectious in *Culicoides* than the AHS virion. These findings seem to indicate that VP2 and VP5 play an important role in mammalian host infectivitiy (Marchi *et al.*, 1995).

1.3.2. The dsRNA genome of AHSV

Ten dsRNA segments compose the genome of the AHSV (Bremer, 1976). The genome is distinct but comparable with the BTV genome. The 10 segments are named by their order of migration: S1-S10 (Figure 1.1). Each of the segments encodes at least one AHSV specific protein (Table 1.2) (Roy *et al.*, 1994b). All the 5' and 3' ends of the AHSV genome segments have characteristic conserved consensus hexamers. The 5' ends consist of the 5'GUU(A/U)A(A/U)-3' and the 3' ends consist of the 5'-(C/A)C(U/A)UAC 3' consensus sequences (Mizukoshi *et al.*, 1993).

1.3.3 AHSV proteins

Seven structural proteins (VP1; VP2; VP3; VP4; VP5; VP6 and VP7)(Figure 1.2) and 4 non-structural proteins (NS1; NS2; NS3 and NS3A) are encoded by the AHSV genome. A variable amount of molecular information is available on the proteins and their individual functions. At least one copy of all AHSV proteins has been cloned and sequenced. Functions have been allocated to each of the virus proteins. Not all functions have however been demonstrated. The proteins and their allocated functions will be discussed in the following section. Where appropriate the functions of the

AHSV proteins are compared or elucidated by comparison with BTV, reovirus or rotavirus proteins and their functions.

Table 1.2: The AHSV genome and the proteins encoded by the specific genome segments (Maree *et al.*, 1998b) (modified)

Segment	Serotype	Base-pairs	Encoded protein	Protein length (aa)	Predicted mol wt	Location
1	9	3965	VP1	1305	150292	inner core
2	3	3221	VP2	1057	123063	outer shell
3	4	2792	VP3	905	103269	outer core
4	4	1978	VP4	642	75826	inner core
5	9	1566	VP5	505	56771	outer shell
6	9	1748	NS1	548	63377	non-structural
7	9	1167	VP7	349	37916	outer core
8	9	1166	NS2	365	41193	non structural
9	3	1169	VP6	369	38464	inner core
10	9	756	NS3/ NS3A	217/ 206	23659 / 22481	non-structural

1.3.3.1. Non-structural proteins NS1, NS2 and NS3/NS3A

During virus infection the AHSV genome encodes at least 3 non-structural proteins besides the 7 structural virus proteins that form the virion. These non-structural proteins have been extensively studied. Their exact function and mode of action is however still not completely clear. Indications are that NS2 and NS3 are involved with virus assembly and release (Roy *et al.*, 1994b).

Non-structural protein NS1 is a 63.122K protein encoded by the genome segment 6 of the AHSV. The proteins assemble into tubules in the infected cell (Huismans & Els, 1979, Maree & Huismans, 1997, Roy *et al.*, 1994b) their function is however still unclear. The NS1 protein is conserved amongst the different AHSV serotypes. A 95-96% amino acid identity is observed. The amino acid similarity of the NS1 between AHSV, BTV and EHDV proteins is significantly lower than the similarity amongst the AHSV NS1 proteins. Amino acid comparisons of the above mentioned NS1 proteins show that only certain regions spanning 10-20 amino acids are conserved up to 70% (Maree & Huismans, 1997, Roy *et al.*, 1994b). These differences seem to be reflected in the difference in appearance of AHSV, BTV and EHDV tubules. AHSV tubules have the appearance of a 'cross hatch' internal structure with smooth sharply defined edges (Huismans & Els, 1979, Maree & Huismans, 1997). This differs from the segmented ladder appearance of the BTV (Huismans & Els, 1979, Urakawa & Roy, 1988) and EHDV tubules (Maree & Huismans, 1997, Nel & Huismans, 1991). No specific function has been allocated to the orbivirus tubules formed by the assembly NS1 proteins (Roy *et al.*, 1994b).

The non-structural protein NS2, encoded by dsRNA segment 8, is 41.197K in size. NS2 is the major protein involved in the formation of virus inclusion bodies (VIB's) (Roy *et al.*, 1994b). The NS2 protein is often associated with the isolated AHSV virion suggesting an association with the virus in cells (Burroughs *et al.*, 1994). Studies on the closely related BTV NS2 protein suggest that the NS2 protein binds ssRNA (Huismans *et al.*, 1987b). It was demonstrated that multimeres of AHSV NS2 form complexes with ssRNA (Uitenweerde *et al.*, 1995). Studies of EHDV NS2 have supported this finding by the identification of ssRNA specific binding motive (Theron *et al.*, 1996). Further studies on BTV NS2 have shown the affinity of the NS2 protein for viral RNA (Theron & Nel, 1997). Phosphorilation of NS2 seems to play a role in the efficiency of orbivirus NS2 to bind ssRNA. This suggests that a cellular kinase may play a role in the NS2 binding ability and therefore may be important in controlling orbivirus replication in cells (Theron *et al.*, 1994). This evidence suggests that the NS2 protein plays a role in virus assembly in the infected cell.

Segment 10 encodes for two non-structural proteins NS3, 24K and NS3A, 23K from two in phase initiation codons (van Staden & Huismans, 1991). A model of the NS3 protein shows intracellular membrane spanning regions of the protein and the termini of the protein both situated in the cytoplasm of the infected cell. Similar models have been proposed for both EHDV and BTV NS3 proteins (van Staden *et al.*, 1995). The function of the NS3 protein has been proposed to be similar to that of the rotavirus NS28 (Both *et al.*, 1994, Desselberger & McCrae, 1994), namely the binding of shelled viruses mediating budding from the endoplasmic reticulum for virus maturation (van Staden *et al.*, 1995) and also possible virus release from the cell (Roy *et al.*, 1994b).

1.3.3.2 Minor core proteins VP1, VP4 and VP6

The core of the AHSV consists of three minor core proteins, VP1, VP4 and VP6 and 2 major core proteins, VP3 and VP7. An enzymatic activity has been assigned to each of the minor core proteins. These activities however still have to be demonstrated.

The largest genome segment encodes the VP1 protein of AHSV. VP1 is also the largest protein of the AHSV 150K (Vreede & Huismans, 1998). The VP1 gene of AHSV-9 has recently been cloned, characterised and the VP1 protein translated *in vitro*. The protein amino acid sequences show high conservation with cognate BTV proteins. The AHSV VP1 protein also contains three regions suggesting possible RNA dependent RNA polymerase activity. In BTV it was shown that VP1 facilitated elongation of RNA in the presence of a poly-(U) template when provided with a poly-A primer. This supports the designated RNA dependent RNA polymerase function of orbivirus VP1 protein (Vreede & Huismans, 1998). Equivalent rotavirus protein, VP1 also has a suggested RNA dependent RNA polymerase activity (Both *et al.*, 1994).

The dsRNA AHSV genome segment 4 encodes VP4, a 75.826K protein (Mizukoshi *et al.*, 1993) with a possible guanylyl transferase activity (Roy *et al.*, 1994b). The BTV VP4 protein binds GTP. The $\lambda 2$ protein of reovirus (Nibert, 1998) and the VP3 protein of rotavirus (Table 1.3) (Both *et al.*, 1994) were shown to have guanylyl

transferase activity (Le Blois *et al.*, 1992). VP4 of BTV forms dimers through a leucine zipper, probably essential for the assembly of BTV core particles (Ramadevi *et al.*, 1998).

The third minor core protein, VP6 of AHSV is encoded by segment 9 and has a predicted size of 38K but migrates at 46K on an SDS PAGE gel (Turnbull *et al.*, 1996). The VP6 of BTV binds ssRNA and dsRNA. Helicase motives found on the protein suggest the helicase activity in both BTV (Roy *et al.*, 1994b) and AHSV VP6 (Turnbull *et al.*, 1996). Besides the possible helicase function, the VP6 has ATPase function that seems to be stimulated by the binding of ssRNA or dsRNA. This suggests that the VP6 protein may aid in the collection of the precise number of mRNA and dsRNA and therefore plays a role in the core assembly of the virus (Stauber *et al.*, 1997).

1.3.3.3 Major core proteins VP3 and VP7

The outer core of AHSV consist of VP3 and VP7. VP3, a protein with the molecular weight of 103.369K, is encoded by dsRNA segment 3 of AHSV. The VP3 protein, the innermost capsid protein of AHSV, is highly conserved and contains group specific antigenic determinants (Roy *et al.*, 1994b, Roy & Sutton, 1998). Baculovirus expressed VP3 and VP7 assemble into CLP's in insect cells (Maree *et al.*, 1998a) similar to CLP's formed by BTV VP3 and VP7. During the formation of BTV core particles the minor core proteins together with VP3 form a subcore onto which the VP7 trimers can assemble (Grimes *et al.*, 1998). In BTV the VP3 and VP7 proteins form the scaffolding for the VP5 and VP2 proteins to bind to. VP2 and VP5 do not assemble spontaneously to form the outer capsid of the virion (Roy, 1992) (Figure 1.2).

The major capsid protein of the inner core of AHSV is VP7. VP7 is a 38.107K protein encoded by segment 7 of AHSV dsRNA genome. The VP7 protein is a group specific antigenic determinant (Roy *et al.*, 1994b) and has been shown to induce an immune response protecting mice against heterologous AHSV serotype challenge (Wade-Evans *et al.*, 1997). The protein is highly conserved amongst orbiviruses and is rich in hydrophobic amino acids. The group specific antigens, VP6 of Rotavirus (Both

et al., 1994, Kohli *et al.*, 1992) and the λ2 and μ1 proteins of reovirus (Nibert, 1998) have been identified as possible analogs to the orbivirus VP7 protein (Table 1.3). The AHSV VP7 protein forms a trimeric structure similar to the VP7 trimer found in BTV (Basak *et al.*, 1996). Baculovirus expressed VP7 protein form hexameric crystals (Chuma *et al.*, 1992), similarly to the crystals also found in AHSV infected cells and cells expressing the VP6 rotavirus protein (Both *et al.*, 1994). These crystals may be a byproduct of virus assembly due to high expression levels in the infected cells or have an otherwise unknown function (Burroughs *et al.*, 1994). VP7 has also been identified as the possible cell attachment protein in *Culicoides variipennis* cells (Xu *et al.*, 1997). The VP7 protein structure and its position in the BTV core is known (Grimes *et al.*, 1998). Further research is however necessary to determine the exact function or functions of the VP7 protein of AHSV.

Table 1.3: Table of Reovirus, Rotavirus and Orbivirus proteins (Tyler & Fields, 1990)

Outer capsid proteins	Core proteins	Non-structural proteins
Reovirus		
σ3; μ1C	λ1; σ2; λ2 (core spike)	μNS; σNS
Minor: σ1	Minor: λ3; μ2	σ1s
Orbivirus		
VP2; VP5	VP3; VP7	NS1; NS2; NS3/NS3a
	Minor: VP1; VP4; VP6	
Rotavirus		
VP4; VP7	VP2; VP6	NS53; NS34; NS35; NS26
Minor: VP5; VP10; VP10c	Minor: VP1; VP3	

1.3.3.4 Major capsid proteins VP2 and VP5

The principal serotype specific antigen and the major component of the outer capsid is the VP2 protein of AHSV. The protein is encoded by segment 2 of the AHSV genome and is 124K in size (Roy *et al.*, 1994b). The presence of neutralising epitopes on the VP2 protein was demonstrated with neutralising monoclonal antibodies (Burrage & Laegreid, 1994). Immunological experiments demonstrate that VP2 confers protection against AHSV serotype specific infection (Martinez-Torrecuadrada *et al.*, 1994, Roy *et al.*, 1996, Stone-Marschat *et al.*, 1996). The VP2 protein is highly

variable amongst different AHSV serotypes as well as other orbivirus VP2 proteins. The C-terminal seems to be most conserved and may be the region of interaction with the more conserved virus proteins VP5 and VP7 (Roy *et al.*, 1994b, Vreede & Huismans, 1994). The VP2 protein of AHSV is implicated in cell attachment of the virus. This, along with to its status as neutralising antigen, supports the findings that the VP2 protein may play a role in virulence determinance (O'Hara *et al.*, 1998)

The second outer capsid protein, VP5 is a 56.793K protein is encoded by segment 5. Although the protein is situated on the outer capsid there is no evidence to show neutralising epitopes on the VP5 protein. It seems that the protein is not as exposed to the outside of the virus as the VP2 protein is (Roy *et al.*, 1994b). The VP5 protein is highly conserved amongst the AHSV serotypes with an amino acid identity of 92% and an amino acid similarity 96% of between AHSV-9 and AHSV-4 VP5 proteins (du Plessis & Nel, 1997). Truncated versions of AHSV VP5 proteins have been described, but it is unknown if the truncated proteins are due to the internal initiation site or due to protease action. The specific function, other than structural, is also unknown (Roy *et al.*, 1994b). The VP5 protein does however seem to play a role in enhancing the immune response against AHSV in the presence of the VP2 protein (Martinez-Torrecuadrada *et al.*, 1996). Mertens (1989) suggested that the VP5 protein may interact with the VP2 protein thereby affecting the VP2 protein conformation possibly enhancing the presentation of neutralising epitopes (Mertens *et al.*, 1989). These suggestions are supported by findings that in the presence of VP5 the protective immune response elicited by VP2 is enhanced (Huismans *et al.*, 1987a, Inumaru & Roy, 1987, Martinez-Torrecuadrada *et al.*, 1996). The VP5 protein however does not seem essential for eliciting protective immune response (Huismans *et al.*, 1987a, Martinez-Torrecuadrada *et al.*, 1996). The VP5 protein has also been linked to the possible determination of virulence of AHSV (O'Hara *et al.*, 1998) establishing its importance as part of the AHSV virion.

The rotavirus proteins VP7 and VP4 (Both *et al.*, 1994) and reovirus σ3 and σ1 (Nibert, 1998) (Table 1.3) fulfill similar functions as the orbivirus VP2 and VP5 proteins. Rotavirus VP7 and VP4 (Conner *et al.*, 1994) and σ1 of reovirus (Virgin *et al.*,

1998) are serotype specific antigens and VP7 and VP4 of rotavirus have been shown to induce neutralising antibodies (Ramig, 1997). The outer capsid proteins of orbiviruses, reoviruses and rotaviruses are therefore the primary focus in the development of new generation vaccines.

1.4. Vaccine development

During the last 200 years, since Edward Jenner's first immunisation in 1798 (Cox, 1997, Levy *et al.*, 1993) , vaccination has become an important method in controlling disease in both humans and animals. During the last century new methods of vaccination have been developed and improved. These include the 'Jennerian' or live viral vaccine, the inactivated vaccine, various subunit vaccines including live viral vaccines (Cox, 1997, Levy *et al.*, 1993) and most recently the nucleic acid vaccines (Robinson, 1997). For each of the types of vaccines both advantages and disadvantages exist. To develop vaccines that are safe, potent, efficacious and available to control a specific disease (House, 1993b) is a continuous process. In this section the different types of vaccines will be examined with regard to vaccines developed against mammalian *Reoviridae*, with emphasis on the Orbiviruses, the AHSV and the BTV, as well as rotavirus vaccine development.

1.4.1 Live virus vaccines.

Live viral vaccines represent the oldest approach to vaccination. Live attenuated or reassorted viruses are used for vaccination (Burrage & Laegreid, 1994, House, 1993b, Murray & Eaton, 1996, Offit *et al.*, 1994). Attenuated viruses are either selected from avirulent strains occurring naturally or viruses attenuated by passage in the laboratory though tissue culture or mouse brain (Burrage & Laegreid, 1994, House, 1993b, Murray & Eaton, 1996). Live viral vaccines are the primary source of current vaccination programmes against mammalian *Reoviridae* infection (Bishop, 1993, Burrage & Laegreid, 1994, House, 1993b, House, 1998, Murray & Eaton, 1996). Live attenuated virus vaccines passaged through tissue culture or mouse brains are currently used in South Africa for vaccination against AHSV and BTV (House, 1998).

These vaccines are credited for keeping potential AHSV epidemics under control (Coetzer & Erasmus, 1994, Meiswinkel, 1998). Reassorted virus vaccines for vaccination against rotavirus infections were developed by reassorting animal and human rotavirus. Live animal rotaviruses, antigenically related to human rotavriuses, but known to be avirulent in humans were used for reassortment live viral vaccines. Selection of desired reassorted rotaviruses containing 10 dsRNA segments of the animal rotavirus together with a human VP7 specific encoding gene of each of the four epidemiologically important VP7 protein of human rotaviruses was achieved. The reassorted animal viruses carried human VP7 proteins. Vaccination trials in children demonstrated protective immune response against human rotavirus infection. Protection dropped after one year making annual vaccination necessary (Bishop, 1993).

Live viral vaccines are advantageous because a very small dosage elicits the most comprehensive immune response. However many disadvantages with the use of live attenuated viral vaccines have been experienced. Some of the potential problems are ineffective attenuation, possibilities of reversion back to virulent virus strains and potential recombination with wild type viruses (Levy *et al.*, 1993). Furthermore possible transmissions by natural vectors make live attenuated vaccines unsafe for use in non-endemic regions (House, 1998).

Live viral vaccines are important in controlling mammalian *Reoviridae* diseases. Potential problems experienced with live vaccines however have required the development of other vaccine types such as the inactivated viral vaccines, subunit viral vaccines and nucleic acid vaccines.

1.4.2 Inactivated viral vaccines.

Inactivated virus vaccines have the advantage over live virus vaccines because there is no need to identify naturally occurring avirulent virus or to attenuated virus in the laboratory. The risk of reversion back to virulence is also eliminated (Bishop, 1993). These advantages however do not always outweigh the disadvantages

experienced with inactivated virus vaccines. The inactivated vaccines are less effective than live viral vaccines and incomplete inactivation could have disastrous effects.

The inactivated BTV vaccines' dosage has to be increased at least 100 fold and two vaccinations are necessary to achieve protective immunity against a single serotype (Murray & Eaton, 1996). Live attenuated virus vaccines elicit a higher neutralising antibody titer then inactivated BTV vaccines (Campbell, 1985). Estes and (1989) report similar findings with rotavirus inactivated vaccines. Live virus vaccines also appear more effective than inactivated virus vaccines against rotavirus infection. A possible reason may be that inactive rotavirus vaccines do not elicit a CTL response whereas for effective protection against rotavirus infection both a humoral and a cytotoxic T cell (CTL) response is necessary (Chen *et al.*, 1997).

In countries where the BTV or AHSV are endemic, no inactivated vaccines are currently used (Murray & Eaton, 1996). In Europe however, a non-endemic region for AHSV, inactivated vaccine against AHSV serotype 4 is available for control of AHSV-4 infection (House, 1993b, House, 1998).

The development of subunit and other new generation vaccines offer alternative solutions to the use of live or attenuated vaccines. These vaccines may provide alternative means to overcome problems experienced with both the live attenuated and inactivated virus vaccines.

1.4.3 Subunit virus vaccines

The antigenicity and the antigenic proteins of mammalian *Reoviridae*, in particular the orbiviruses (BTV and AHSV) and the rotaviruses are extensively studied. Serogroup and serotype specific antigenic proteins have been identified and form the basis of subunit vaccine development. Critical to the development of subunit vaccines is the selection of the appropriate target epitopes or antigens (Virgin *et al.*, 1994) which are presented in the best possible way. In the following sections the main approaches

of subunit vaccine development against BTV, AHSV and rotavirus infection will be discussed.

1.4.3.1 Recombinant proteins as subunit virus vaccines

The development of recombinant proteins as subunit vaccines for orbiviruses such as BTV and AHSV are primarily based on the serotype specific antigen VP2 (Huismans *et al.*, 1985, Martinez-Torrecuadrada & Casal, 1995, Murray & Eaton, 1996, Roy *et al.*, 1996, Stone-Marschat *et al.*, 1996, Urakawa *et al.*, 1994). In the case of rotavirus VP7, VP4 and VP6 are involved (Bishop, 1993, Conner *et al.*, 1994, O'Neal *et al.*, 1997). The following section will deal with some of the advantages and disadvantages as well as the successes and failures of using recombinant expressed proteins as subunit vaccines against orbivirus or reovirus infection.

Eukaryotic expression systems are primarily used for orbivirus or reovirus recombinant protein expression (Conner *et al.*, 1994, Emslie *et al.*, 1995, Martinez-Torrecuadrada *et al.*, 1994, Martyn *et al.*, 1994, Vreede & Huismans, 1994). Prokaryotic expression systems have also been investigated for expression of subunit vaccines. Unfortunately very poor immune responses against these proteins have been elicited (Bishop, 1993, Conner *et al.*, 1994). This may be due to the extreme conformational sensitivity of rotavirus VP7 and orbivirus VP2 proteins. For the development of subunit vaccines against orbiviruses and mammalian reoviruses, posttranslational modifications may play an important role in the development of suitable subunit vaccines. The most frequently used eukaryotic expression system for rotavirus and orbivirus protein expression is the baculovirus expression system (Conner *et al.*, 1994, Martinez-Torrecuadrada *et al.*, 1994, Vreede & Huismans, 1994).

Alternative expression systems such as yeast (Martyn *et al.*, 1994) and the simple eukaryotic amoebae, *Dictyostelium discoideum* have also been investigated (Emslie *et al.*, 1995) with variable results. Yeast expressed VP2 of BTV did not elicit a protective immune response (Martyn *et al.*, 1994). More recently VP7 of rotaviruses was expressed in the simple eukaryote *Dictyostelium discoideum*, an inexpensive

eukaryotic expression system. Initial results indicate the expressed VP7 was successfully N-glycosilated and the VP7 protein reacted with neutralising monoclonal antibodies (Emslie *et al.*, 1995).

The baculovirus system is still the norm for protein expression in the development of subunit virus vaccines until alternative systems are proven reliable in subunit vaccine development. The posttranslational modifications often necessary for the correct protein structure and antigenicity of proteins is facilitated by this baculovirus expression system. An additional advantage is the potential for co-expression of a variety of proteins. This is an important tool to form virus structures or to enhance the conformation of recombinant proteins. (Roy *et al.*, 1994b, Roy & Sutton, 1998)

Baculovirus expressed recombinant VP2 proteins of both AHS and bluetongue viruses have been demonstrated to induce serotype specific protective immune response in host animals (Marshall & Roy, 1990, Roy *et al.*, 1996). A more effective and increased protective immune response was achieved when co-expressed VP2 and VP5 of AHSV (Martinez-Torrecuadrada *et al.*, 1996) or VP2 and VP5 of BTV (Mertens *et al.*, 1989) were used for vaccination. Similar observations were made in the rotavirus subunit vaccine development against rotaviruses. The rotavirus VP7 (Estes & Cohen, 1989) and VP4 proteins expressed without other virus proteins in the baculovirus system showed rather poor antigenic properties (Bishop, 1993, Conner *et al.*, 1994). Dormitzer (1992) demonstrated protective antigenic immune response against the VP7 of rotaviruses is indeed conformational dependent. The protein is also and dependent on the presence of other virus proteins (Dormitzer *et al.*, 1992). Maintaining neutralising epitopes on these antigens seems to be conformational dependent (Bishop, 1993).

Some of the serogroup specific antigens such as VP7 of AHSV have been shown to induce protective immune response mice (Wade-Evans *et al.*, 1997). Rotavirus VP1 and VP6 induce $CD8^{+}$T lymphocyte response but their involvement in the protection against rotavirus infection has not yet been demonstrated (Bishop, 1993, O'Neal *et al.*, 1997).

A more wholelistic approach to subunit virus vaccines may be the use of core like particles (CLP's) or virus like particles (VLP's). These VLP's present virus proteins in conformation close to the live virus but have the advantage of being non-replicative. CLP's of BTV were formed by the co-expression of the VP3 and VP7 proteins of BTV using a baculovirus expression system. The immunogenicity of the CLP's were tested in sheep, viremia and neutralising antibodies developed after vaccination. Challenge with homogenous BTV resulted slight clinical lesions and fever, however protection against BTV was achieved (Roy 1996). CLP's against the AHSV have also been produced (Maree *et al.*, 1998a) but have not been evaluated as possible subunit vaccines.

The co-expression of proteins of BTV or rotavirus has resulted in the formation of virus like particles (VLP's). VLP's of BTV have also been produced by the co-expression of the four major structural virus proteins VP2, VP5, VP3 and VP7 of BTV (French & Roy, 1990). Vaccination with the BTV VLP's elicited strong immune response of serotype specific antibodies in Guinea pigs. Vaccination of sheep with BTV VLP's resulted in complete protection against challenge with BTV. Low levels of protection against heterologous BTV challenge were also recorded (Roy, 1992, Roy, 1996, Roy *et al.*, 1994a, Roy *et al.*, 1992, Roy & Sutton, 1998). Rotavirus VLP's were generated by various combinations of baculovirus co-expressed VP2, VP6 and VP7 rotavirus proteins. VLP's consisting of VP2, VP6 and VP7 or only VP2 and VP6 were formed. Mucosally administered VLP's consisting of VP2 and VP6 elicited protective VP6 specific IgA antibodies. VLP's containing VP2 and VP6 may be a safe, easy to administer potential virus vaccine against rotavirus infection (O'Neal *et al.*, 1997).

Recombinant proteins for the use as virus vaccine is currently a very ineffective and expensive way of producing subunit vaccines against orbivirus or rotavirus infections. The amount of antigen needed for effective protection against infection is extremely high relative to the live attenuated vaccine dosage and baculovirus expression system very expensive. The subunit vaccine seems to be a far way from becoming commercially viable for vaccination against orbiviruses and rotaviruses.

However the research on recombinant proteins as subunit vaccines may be of great value in the development of other potential subunit vaccines. For example the use of virus vectors for the presentation of the antigenic protein in the vaccinated animals or humans.

1.4.3.2 Virus vector subunit virus vaccines.

Attenuated virus systems have been extremely well studied and have the potential to be developed as virus vectors presenting foreign antigenic determinants. The vaccinia virus is an ideal experimental candidate for research in this direction. Production of the virus is cost effective and the virus is stable when lyophilized. Vaccination is easy and a scar, the result of vaccination, can serve as proof of immunisation (Levy *et al.*, 1993). The outer capsid protein of rotavirus, VP7 cloned into the vaccinia virus genome was inoculated into mice. The recombinant VP7 specific vaccinia virus induced a CTL immune response lysing cells infected with heterologous rotavirus. These initial experiments demonstrated the potential of live attenuated virus vectors for protection against infection with rotaviruses (Offit *et al.*, 1994). Vaccinia expressed AHSV-4 VP2 protein induced protective neutralising antibodies in horses resulting in total resistance against homologous virus challenge (Stone-Marschat *et al.*, 1996). VP7 of BTV conferred partial protection against BTV challenge when administered as recombinant BTV VP7 specific capripox virus vaccine (Wade-Evans *et al.*, 1996). Adenovirus could also be investigated as alternative to pox virus vectors for orbivirus or rotavirus vaccination. (Conner *et al.*, 1994).

1.4.4 Viral peptide vaccines.

In the previous sections the focus was in subunit vaccines development using recombinant antigenic proteins to induce protection. Viral peptide vaccines were developed as a result of a better understanding of important serotype specific epitopes. Two approaches can be followed: The expression of these antigenic regions as recombinant peptides or the synthetic manufacturing of the peptides for vaccination purposes.

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VP4 of rotavirus contain potential linear neutralising epitopes to be used as peptide vaccines. Synthetic peptides of VP4 react with neutralising monoclonal antibodies against rotaviruses and therefore show potential as vaccines (Bishop, 1993). It was shown that synthetic peptides of VP7 and VP4 linked to carrier proteins could induce anti-rotavirus antibody response protecting against virus infection, neutralising rotavirus *in vitro* (Ijaz *et al.*, 1995). In AHSV such a potential region may have been identified on the VP2 protein (Martinez-Torrecuadrada & Casal, 1995). Further studies on the functioning of the synthetic peptide vaccines still have to be investigated and future research on their applications and suitability as virus vaccines to take place.

1.4.5 Nucleic acid vaccines

A new approach in controlling infective agents is the use of nucleic acid vaccines. These novel vaccines are both easy to construct and to produce and have the potential for a wide range of applications. Experiments have shown that vaccination with DNA does not only induce the formation of antibodies but also stimulates the cytolytic T cell (CTL) protective immune response. (Barry & Johnston, 1997, Robinson, 1997). Nucleic acid vaccination can be administered by a gene gun, injection intramuscularly (Barry & Johnston, 1997) or most recently by oral immunisation (Chen *et al.*, 1997). Although gene immunisation method may play an important role in nucleic acid vaccination the efficiency of the immunisation primarily relies on the character of the antigen and the genotype of the host organism (Barry & Johnston, 1997). No successful attempt of nucleic acid vaccination against orbiviruses has been published.

Nucleic acid vaccines for protection against rotavirus infection have been successfully demonstrated in mice. Plasmids carrying VP7, VP4 or VP6 of rotavirus were used for vaccination by injection into mice. These mice were protected against rotavirus challenge. Both humoral and CTL immune responses were elicited (Chen *et al.*, 1997). More recently VP6 rotavirus plasmid DNA was successfully administered

b. 14442991
i. 14746591

orally by protecting the nucleic acid with a poly(lactide-coglycolide) (PGL) envelope. Protective immune response was elicited against rotavirus challenge in mice (Chen *et al.*, 1998).

Nucleic acid vaccination may play an important part in the development of vaccines against viruses especially when effective oral administration of the vaccines has been developed.

1.5 Conclusion and Aims

AHS is a serious potential problem in Sub- Saharan Africa. Outbreaks may result in great economic loss. It is therefore important to manage the AHS disease effectively to minimise outbreaks and potential epidemics.

Currently a live attenuated AHSV vaccine is used commercially to protect against AHS. This vaccine has been used very successfully and it has been credited with the prevention of a potential AHSV epidemic in South Africa during 1996 (Meiswinkel, 1998). There are however many problems related to the use of a live attenuated virus vaccine. Therefore a number of alternative vaccines are under investigation as candidates for new generation vaccines against AHSV infection. Most of these are focused on the use of the outer capsid VP2 protein as a subunit vaccine. The AHSV-4 VP2 recombinant protein has been shown to elicit serotype specific neutralising immune response in horses. This vaccine protected the horses against homologous AHSV challenge therefore confirming the suitability of the VP2 protein.

The live attenuated virus vaccine, produced by Onderstepoort Biological Products, protect against all serotypes by presenting a cocktail of serotypes of AHS viruses. Although there is linked crossprotection of some serotypes (e.g. 3 and 9) (Coetzer & Erasmus, 1994) the serotypes in general are defined by the specificity of their neutralisation specific immune response. A subunit vaccine will therefore have to consist VP2's of most, if not all the serotypes. Alternatively, methods of stimulating a more heterologous protective immune response should be investigated. There is

however as yet very little evidence that this could be achieved successfully. Evidence from rotavirus vaccine development (as indicated in the introduction) indicates an improved antigenicity of the serotype specific antigen if other virus proteins are present. AHSV VP7 a serogroup specific antigen has been demonstrated to induce protective immune response in mice. The combination of the serogroup and serotype specific antigens in a VLP should therefore not only enhance the antigenic properties of VP2 but also provide for a more well rounded protective immune response. Indications are that the eliciting of a neutralising immune response against AHSV induced by VP2 can be enhanced by VP5 of the same serotype.

In our laboratory AHSV-3 VP2 proteins are expressed in high quantities in insect cells. Most of these VP2 proteins were however found to be insoluble and did not effectively elicit a neutralising immune response. In our investigation we will therefore focus on ways to increase the serotype specific immunogenicity of the baculovirus expressed VP2 protein. Two possible solutions were investigated to increase the solubility and therefore possibly the immunogenicity of VP2. Firstly chemical solubilisation of the aggregated VP2 followed by a refolding process was investigated. Secondly co-expression of the two outer capsid proteins VP2 and VP5 was attempted. This approach required the characterisation of the VP5 gene of AHSV-3 and the optimization of the expression of the VP5 protein insect cells. Previous attempt to express the VP5 gene of AHSV-9 resulted in disappointingly low expression levels. The Bac-to-Bac™ expression system with which other AHSV proteins had been expressed very effectively was selected for the VP5 expression. For similar reasons the Bac-to-Bac™ dual expression system was also used for the first time to express two AHSV outer capsid proteins VP2 and VP5 of AHSV serotype 3.

CHAPTER 2

Characterisation and chemical solubilisation of recombinant baculovirus expressed AHSV-3 VP2

2.1 Introduction

The outer capsid of the AHSV virion consists of two major proteins VP2 and VP5 (Roy *et al.*, 1994b). The VP2 protein is the primary serotype specific antigen of the orbiviruses (Burrage *et al.*, 1993, Martinez-Torrecuadrada *et al.*, 1994). Soluble BTV VP2 proteins have been shown to induce serotype specific protection against BTV challenge (Huismans *et al.*, 1987a). The VP2 protein of orbiviruses is therefore the best, if not only candidate for a subunit vaccine. Baculovirus expressed BTV VP2 protein induces serotype specific antibodies (Inumaru & Roy, 1987, Urakawa *et al.*, 1994). More recently soluble AHSV-4 VP2 expressed by means of a baculovirus recombinant was shown to elicit protective, neutralising antibodies in horses that provided protection against AHSV-4 challenge (Roy *et al.*, 1996, Stone-Marschat *et al.*, 1996). Neutralising antibodies have also been elicited against baculovirus expressed AHSV-3 VP2 (Vreede & Huismans, 1994). The level of expression and immune response was however rather poor. A significantly increased level of AHSV-3 VP2 expression was achieved by Grant Napier (personal communication) with the use of the Bac-to-Bac™ baculovirus expression system (Luckow *et al.*, 1993). Initial vaccine trials in horses unfortunately elicited no detectable neutralising immune response against the Bac-to-Bac™ expressed VP2 proteins. However in this department no attempt was made to separate the soluble from the insoluble VP2. Subsequent experiments indicated that most, if not all of the VP2 was in an aggregated, insoluble form. This problem raised questions about the conformation of the VP2 expressed at such high levels in insect cells.

In our laboratory it was determined that the VP2 protein harvested late in the baculovirus infection cycle was predominantly in an aggregated form. Aggregation of

proteins often occurs if high levels of proteins are expressed in a heterologous expression system (Jaenicke & Seckler, 1997, Neugebauer, 1990, Roy & Jones, 1996, Rudolph & Lilie, 1996). Aggregation is a co-operative process and is often advantageous for the cells expressing the foreign protein (Jaenicke & Seckler, 1997). Other factors such as high or low temperatures, ionic concentration or pH may also play a role in protein aggregation (Franks, 1995).

Aggregated proteins can be de-aggregated by solubilisation with the use of detergents. The denatured proteins can thereafter often be successfully refolded, particularly proteins aggregated due to a concentration effect (Rudolph & Lilie, 1996). A vast number of detergents are available for de-aggregation of proteins (Neugebauer, 1990). Chaotrophs such Guanidinium HCl (6-8M) and Urea (6-8M) are recommended for a general scheme of de-aggregation of highly hydrophobic proteins (Jaenicke & Seckler, 1997, Neugebauer, 1990, Rudolph & Lilie, 1996) such as AHSV-3 VP2. Guanidinium HCl is the preferred detergent because it is the stronger chaotroph. Urea may also contain isocyanate which can result in carbamylation of free amino-groups of the polypeptides (Jaenicke & Seckler, 1997, Neugebauer, 1990, Shi *et al.*, 1997). Different incubation temperatures as well as the addition of reducing agents may also affect the success of the de-aggregation of the proteins by solubilisation (Neugebauer, 1990, Rudolph & Lilie, 1996). Refolding of the de-aggregated, denatured protein may be achieved by dialysis, dilution (Neugebauer, 1990, Rudolph & Lilie, 1996) or desalting by Sephadex size exclusion chromatography (Determann, 1969). Desalting by size exclusion chromatography involves the separation of protein and solvent. It also facilitates the separation of precipitating proteins from proteins that refold and remain soluble therefore preventing cooperative aggregation process. (Determann, 1969, Neugebauer, 1990) .

The expression levels of the VP2 protein expressed by the new Bac-to-Bac™ expression system were very high in comparison to the expression in the previous baculovirus expression system. These high expression levels of VP2 may be the cause of the aggregation of VP2 insect cells. Other possible causes for aggregation were also investigated. The high level of expression, resulting in the high

concentration of VP2 in the cell, seemed to be the main cause of VP2 aggregation. However, VP2 also seemed sensitive to freezing. An attempt was made to regain soluble VP2 by chemical de-aggregation followed by refolding of the protein by means of desalting.

2.2 Materials and Methods

2.2.1 Insect cell tissue culture.

Insect cells of the Fall Army worm, *Spodoptera frugiperda* (Sf-9) were obtained form the American Type Culture Collection (ATCC). The cells were cultivated in monolayers at 28°C using Grace's insect medium containing L-glutamine and $NaHCO_3$ (Highveld Biological PTY, Ltd), 7ml of antibiotics/500ml Grace's Medium (Penicillin G Na (10mg/ml), Streptomycin Sulphate (10mg/ml)), Fungizone (25μg/ml)) and 10% Fetal Calf Serum (Highveld Biological PTY, Ltd)

2.2.2 Infection of insect cells.

Spodoptera frugiperda (Sf-9) cells are highly susceptible to infection with baculoviruses. The Sf-9 cells were cultivated according to the protocol described by O'Reilly *et al* (1992). For infection of the cells with recombinant baculoviruses, cells were harvested and seeded at a density of $1X10^7$ cells/ 80 cm^3 flask or $1X10^6$ cells/ 35mm well and left to attach to the flask/well for 1h at room temperature (RT). The cells were infected at a multiplicity of infection (m.o.i) of at least one plaque forming unit (pfu) per cell. Medium was added to a final volume of 7ml/80cm^3 flask or 1ml/35mm well. The cells were incubated for 1 hour at 28°C before more medium was added to the final volume of 12ml/80cm^3 flask or 2ml/35mm well. The infected cells were incubated at 28°C for appropriate time. Thereafter the cells were harvested by shaking the attached cells of the plastic surface and concentrating them by centrifugation at 2000g for 3 minutes. The supernatant was discarded and the cells washed in 1XPBS (137mM NaCl; 2.7mM KCl; 4.3mM $Na_2HPO_4.2H_2O$; 1.4mM KH_2PO_4 ; pH 7.3). The cells were pelleted by centrifugation at 2000g for 3 minutes followed by a second washing step in 1X PBS. Finally the cells were resuspended in 1X PBS to a final concentration of $2.5X10^7$ cells/ml before analysis.

2.2.3 Protein gel electrophoresis.

Proteins were analysed by analytical SDS-PAGE gel electrophoresis. Prior to analysis the protein samples were mixed with an equal volume of 2XPSB (0.125M Tris pH6.8; 4% SDS (w/v); 20% glyserol (v/v); 10% 2-mercapto ethanol (v/v)), heated to 95°C for 5 minutes and sonified. The proteins were separated on a 12% or 15% SDS-PAGE gel as described by Sambrook *et al* (1989). The separating gel containing 0.375M Tris-HCl and 0.1% SDS and the 3% stacking gel containing 0.125M Tris-HCl pH 6.8 and 0.1% SDS were prepared of a stock solution of 30% acrylamide and 0/08% bisacrylamide. The gels were polymerized chemically by the addition of 0.008% (v/v) TEMED (N,N,N',N',-tetramethylethelenediamine) and 0.08% (w/v) ammonium persulfate. Electrophoreses was carried out in TGS buffer (0.025M Tris-HCl pH8.3; 0.192M glycine; 0.1% SDS). The gels were stained in 0.05% Coomassie Blue, 50% methanol, 10% glacial acetic acid and destained in 5% methanol, 5% acetic acid for visualisation of the proteins.

2.2.4 Solubility analysis of AHSV-3 VP2 proteins.

Sf-9 cells were cultivated as described in (2.2.1) and infected with recombinant AHSV-3 VP2 baculovirus (2.2.2) provided by Grant Napier. The infected cells were incubated at 28°C and harvested at various times after infection. The harvested cells were resuspended in lysis buffer (0.15M KCl; 10mM Tris; 0.1% Triton X100) as described by Huismans *et al* (1987) and thereafter incubated at RT for 30 minutes before lysis through 1ml syringe with a .22 gauge needle (2.2.7). The lysed cells were centrifuged at 2000 rpm

for 5 minutes in a bench centrifuge. The supernatant was again centrifuged at 10 000g for 1h in the Beckmann ULTRA SW 50.1 rotor. The pellets of both centrifugation steps were resuspended to the original volume in 1XPBS. The proteins in each of the fractions were analysed using SDS PAGE analytical protein electrophoreses (2.2.3).

2.2.5 Lysis of Sf-9 cells by a freeze-thaw method

Harvested, infected Sf-9 cells were resuspended in one of each of the following lysis buffers: 1XPBS (137mM NaCl; 2.7mM KCl; 4.3mM $Na_2HPO_4.2H_2O$; 1.4mM KH_2PO_4 ; pH 7.3) 1%Triton X100; 1X PBS 0.5% Triton X100; 1X PBS 0.1% TritonX100; 1XPBS 0.1% N-Lauryl Sarcosine sodium salt (SIGMA); 0.15M STE (0.01M Tris pH7.4; 0.001M EDTA; 0.15M NaCl) 1% Triton X100; 0.15M STE 0.5% TritonX100; 0.15M STE 0.1% Triton X100; 0.15M STE 0.1% N-Lauryl Sarcosine sodium salt (SIGMA). The cells were subsequently frozen at -20°C and thawed on ice. This procedure was repeated 3X. The lysed cells were centrifuged at 10 000g for 1h in the Beckmann ULTRA SW 50.1 rotor. The pellet was resuspended to the original volume in 1XPBS. Samples of supernatant and pellet were analysed by SDS PAGE gel electrophoresis (2.2.3).

2.2.6 Lysis of Sf-9 cells by dounce mechanism

Harvested, infected Sf-9 cells were resuspended in one of each of the following lysis buffers: 1XPBS (137mM NaCl; 2.7mM KCl; 4.3mM $Na_2HPO_4.2H_2O$; 1.4mM KH_2PO_4 ; pH 7.3) 1%Triton X100; 1X PBS 0.5% Triton X100; 1X PBS 0.1% TritonX100; 1XPBS 0.1% N-Lauryl Sarcosine sodium salt (SIGMA); 0.15M STE (0.01M Tris pH7.4; 0.001M EDTA; 0.15M NaCl) 1% Triton X100; 0.15M STE 0.5% TritonX100; 0.15M STE 0.1% Triton X100; 0.15M STE 0.1% N-Lauryl Sarcosine sodium salt (SIGMA) and incubated for 10 at RT. The cells were mechanically lysed using a 1ml tight dounce apparatus (Wheaton). The lysed cells were centrifuged at 10 000g for 1h in the Beckmann ULTRA SW 50.1 rotor. The pellet was resuspended to the original volume in 1XPBS. Samples of supernatant and pellet were analysed by SDS PAGE (2.2.3).

2.2.7 Lysis of Sf-9 cells by .22 gauge needle and syringe

Harvested, infected Sf-9 cells were resuspended in lysis buffer and incubated for 10 minutes at RT. The cells were mechanically lysed by forcing the cells through a .22 gauge needle on a 1ml syringe. The lysed cells were centrifuged at 10 000g for 1h in the Beckmann ULTRA SW 50.1 rotor. The pellet was resuspended to the original volume in 1XPBS. Samples of supernatant and pellet were analysed by SDS PAGE gel electrophoresis (2.2.3).

2.2.8 Solubilisation of protein by pH and salt concentration

Harvested, infected Sf-9 cells were resuspended in lysis buffer (0.15M KCl; 10mM Tris pH.8; 0.1% Triton X100) and incubated at RT for 30 minutes. The cells were lysed using 0.22 gauge needle on a 1ml a syringe (2.2.7). The lysed cell sample was divided into two equal parts. To the one half, equal volumes of 0.15M STE were added (control). To the second half of the lysed cell sample equal volumes of solubilisation buffer was added to a final concentration of 0.5M $MgCl_2$ and 0.1M MES (2-Morpholino ethane sulfonic acid pH5.0) (Huismans *et al.*, 1987a). Both samples were incubated in ice for 90 minutes before centrifugation at 2000 rpm in bench top centrifuge thereafter centrifuging the supernatant at 10 000g in a Beckmann ULTRA SW 50.1 rotor for 1h. The pellets of both centrifugation steps were resuspended in 1XPBS. Equal amounts of each fraction were analysed by SDS PAGE (2.2.3).

2.2.9 Denaturing of insoluble protein by Urea or Guanidine HCl

Harvested, infected cell were lysed mechanically trough a .22 gauge needle (2.2.7.) in lysis buffer containing 0.15M KCl; 10mM Tris pH.8; 0.1% Triton X100; 40ug/mlDNAse; 20ug/ml RNAse (Lin & Cheng, 1991) . The aggregated protein was collected by centrifugation in a bench centrifuge at 2000 rpm for 5 minutes. The supernatant was decanted and saved as a control. The pellet, containing the aggregated VP2, was resuspened in solubilisation buffer (Guanidine solubilisation buffer pH7.8: 20mM Tris; 0.5M KCl; 5mM Imidasole; 6M Guanidine HCl; Urea solubilisation buffer pH 7.8 : 20mM Tris; 0.5M KCl; 5mM Imidasole; 8M Urea (Shi *et al*, 1997) and incubated at 37°C for 2 hours (Rudolph & Lilie, 1996). The de-aggregated proteins were centrifuged at 2000 rpm for 5 minutes in a bench centrifuge to

remove any insoluble fraction. The supernatant was centrifuged at 10 000g for 1h in the Beckmann ULTRA centrifuge. The pellets of both centrifugation steps were resuspended in 1X PBS and a sample of each fraction was analysed by protein gel electrophoresis (2.2.3).

2.2.10 Protein refolding

Proteins solubilised by 6M Guanidinium HCl (GHCl) were refolded by desalting with the aid of dilution or size exclusion chromatography (Determann, 1969). The soluble, denatured proteins in 6M GHCl were diluted slowly by the addition of 1X PBS to a concentration of 3M followed by a dilution 1.5 M and finally to 0.75 molar. By centrifugation at 10000g in SW 50.1 for 1h it was determined if there was still soluble protein in the sample.

The complete desalting and thereby refolding of the protein was attempted using a small G75 Sephadex size exclusion column. The Sephadex was made up in water according to specifications (Determann, 1969). The column was packed into a Pasteur pipette and equilibrated with 1X PBS and then calibrated with BSA in 1XPBS, BSA in 6M GHCl and finally with only GHCl. Fractions of 100 μl were taken and analysed spectrophotometrically at a wavelength of 280nm. The BSA was used both to calibrate the proteins as well as to decrease any possible unspecific binding (personal communication, Prof. L. Visser) of the AHSV-3 VP2 protein to the column by first saturating the column with BSA. The proteins denatured in 6M GHCl were diluted to a salt concentration of 3M GHCl. The proteins were loaded onto the column and 100μl fractions were collected. The fractions were analysed by spectrophotometry at and at 280nm to determine where the bulk of the protein was situated. Both proteins (Sambrook *et al.*, 1989) and GHCl (Boehringer Mannheim Catalogue) can be measured at this wavelength. The fractions containing mainly protein were pooled and the solubility of the refolded protein was determined by centrifugation at 10000 rpm for 1h in SW 50.1 rotor of the Beckmann Ultra. The soluble fraction was concentrated by freeze drying for analysis. The total soluble and particulate fractions were analysed by SDS-PAGE gel electrophoresis (2.2.3).

2.3 Results

All methods used to obtain the following results were described in section 2.2. unless otherwise stated.

2.3.1 The solubility of VP2 in insect cells at different times after infection

Recombinant baculovirus with the AHSV-3 VP2 gene cloned under the control of the late polyhedrin promoter was obtained form Grant Napier. A preliminary investigation showed that when VP2 was expressed in insect cells and the proteins harvested at 70hpi, VP2 was predominantly in an aggregated and therefore insoluble form. This raised the question if this result was influenced the VP2 concentration in the cell and therefore influenced by the time of harvesting after infection. Protein concentration can affect the solubility of a protein. There was therefore a possibility that the increase in VP2 concentration during the course of the infection cycle may be the cause of VP2 aggregation. To address this question Sf-9 cells were infected with the AHSV-3 VP2 specific recombinant baculovirus and the cells harvested at 18h, 32h,

36h; 42h; 48h and 72h after infection. The cells were subsequently lysed in a buffer containing 0.1% Triton X-100 and fractionated by centrifugation at 2000 rpm in a bench centrifuge for 5 minutes. This was followed by a second centrifugation of the supernatant at 10000g in the SW 50.1 rotor of the Beckmann ULTRA centrifuge. Representative samples of the whole cell fraction, the 2000 rpm pellet, the 10000g pellet and the 10000g supernatant were analysed on a 12 % SDS PAGE gel and visualised with Coomassie brilliant blue staining.

The results are shown in Figure 2.1. The fractions harvested at 18hpi and 32hpi are not shown because the levels of VP2 expression were to low to be detected. Lane 1 in Figure 2.1 shows the increase of total VP2 in insect cells can be observed over the time interval 36hpi to 72hpi. The increased total concentration of the VP2 protein in the cells seems to correlate with the decline in the soluble fraction (lane 4) and an increase in the aggregated VP2 (lanes 2 and 3). At 36hpi soluble VP2 can be observed (lane 4; 36hpi) while at 72hpi no soluble VP2 is visible (lane 4; 72hpi). Throughout the time trial a significant amount of VP2 was always in an aggregated form (lanes 2 and 3) irrespective of the concentration in the cells. The majority of VP2 expressed by means of the Bac-to-Bac™ baculovirus system in insect cells seems to occur in an aggregated form in the insect cells. These results suggest that an increase in VP2 concentration in the cells pushes the equilibrium between soluble and aggregated VP2 into the direction of VP2 aggregation until all VP2 is found an aggregated form. Other factors could however also affect VP2 solubility.

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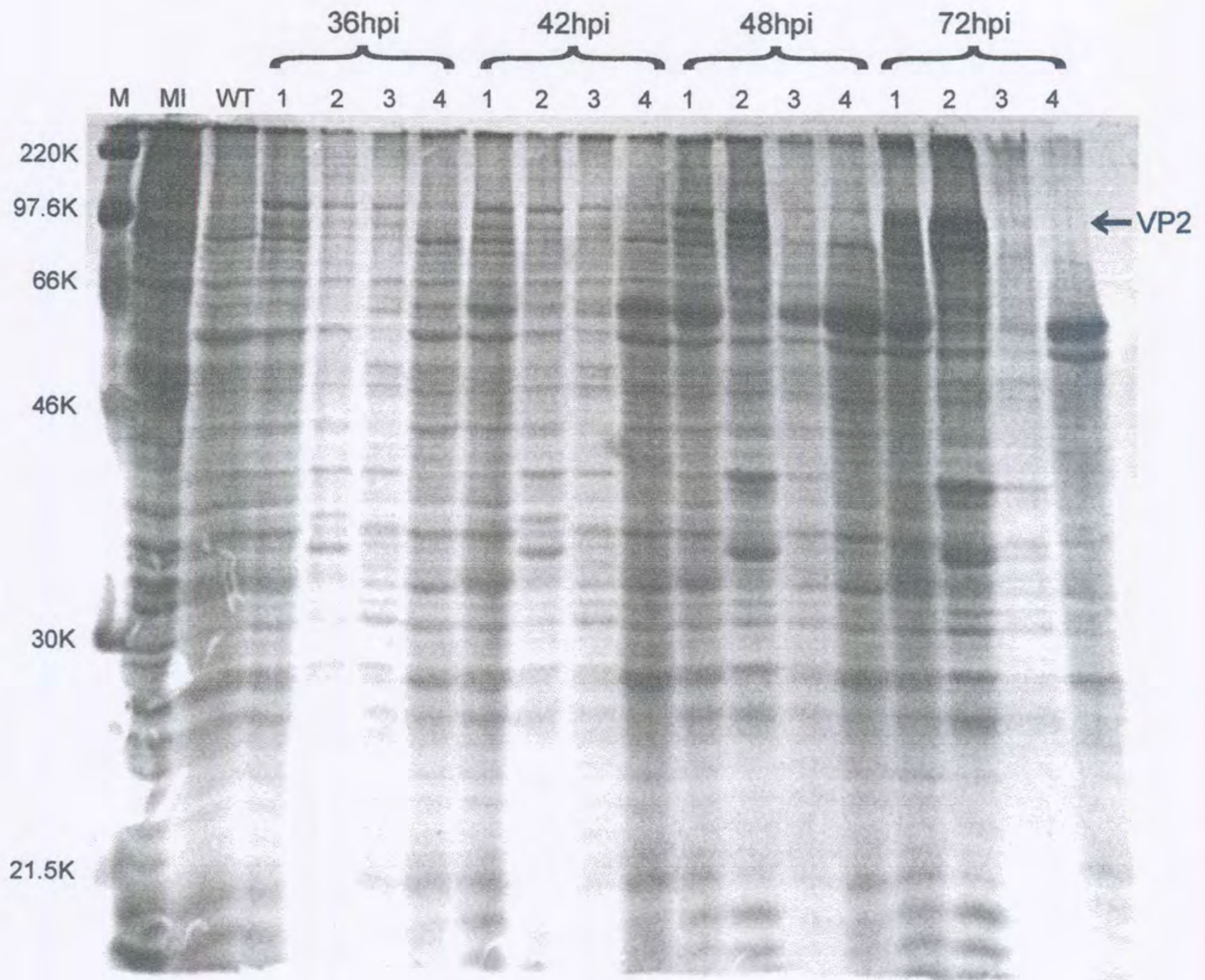

Figure 2.1: SDS PAGE gel analysis of protein solubility in VP2 recombinant baculovirus infected insect cells harvested at different times after infection. The protein fractions were prepared as described in lane 1 to 4 of each time after infection.

M : molecular weight marker
MI : mock infected insect cells
WT: wild type infected insect cells
Each harvesting time after infection (36h, 42h, 48h and 72h) of AHSV-3 VP2 recombinant baculovirus infected cells is represented by four lanes:
Lane 1: Whole cell fraction
Lane 2: 2 000 rpm pellet
Lane 3: 10 000 rpm pellet
Lane 4: 10 000 rpm supernatant

2.3.2 Solubility of VP2 affected by different cell lysis methods

The solubility of VP2 can also be influenced by the method of cell lysis. The method of cell lysis and its effect in the solubility of the baculovirus expressed AHSV-3 VP2 proteins was therefore examined. For this purpose, Sf-9 insect cells expressing VP2 were lysed by a variety of mechanical lysis methods in combination with a variety of lysis buffers. The infected insect cells were harvested at 42hpi. The cells were lysed as indicated in Table 2.1 as described in the methods. After cell lysis, the fraction of soluble VP2 was determined by centrifugation of the lysed cells at 10 000g for 1 hour in the SW 50.1 rotor of the Beckmann ULTRA centrifuge. The supernatant and pellets were analysed by SDS PAGE gel electrophoresis. The results are summerised in Table 2.1.

The results show that the freeze thaw mechanical lysis method combined with a variety of different lysis buffers (Table 2.1) yielded no soluble protein. Both the dounce and syringe methods produced similar results. With detergent concentrations of 0.1% Triton X-100 or 0.1% N-Lauroyl sarcosine Na salt in either 1X PBS or 0.15M STE buffers, a small fraction of the recombinant AHSV-3 VP2 was found to be in a soluble form although the largest fraction of the protein was still aggregated. This indicated that the cell lysis method could influence the solubility of the recombinant AHSV-3 VP2 protein. Freeze thawing as well as detergent concentration of higher than 0.1% seemed to result in VP2 aggregation. The freeze thawing method is commonly used to disrupt cells. In the case of VP2 however, this method seems to have a negative effect on the solubility of VP2 and therefore alternative methods of cell lysis are recommended.

2.3.3 AHSV-3 VP2 solubilisation with pH and high salt concentration

The previous experiments have shown that different factors such as protein concentration as well as the cell the lysis method and the lysis buffer play a role in the solubility of the baculovirus expressed AHSV-3 VP2 protein. It has previously been

Table 2.1: A summary of cell lysis methods and their effect on the solubility of recombinant VP2 protein harvested at 42hpi. Column 1 shows the different buffers used in combination with the different mechanical lysis methods indicated in column 2,3 and 4. The + indicates the relative amounts of VP2 protein either in soluble form or aggregated form.

BUFFERS	FREEZE THAW		DOUNCE		0.22 SYRINGE NEEDLE	
	soluble	aggregate	soluble	aggregate	soluble	aggregate
1x PBS 1.0% TRITON-X 100		+++		+++		+++
1x PBS 0.5% TRITON-X 100		+++		+++		+++
1x PBS 0.1% TRITON-X 100		+++	+	++	+	++
1XPBS 0.1% N Lauryl sarcosine Na Salt		+++	+	++	+	++
0.15M STE 1.0% TRITON-x 100		+++		+++		+++
0.15M STE 0.5% TRITON-x 100		+++		+++		+++
0.15M STE 0.1% TRITON-x 100		+++	+	++	+	++
0.15M STE 0.1% N Lauryl sarcosine Na Salt		+++	+	++	+	++

shown that VP2 could be removed from purified BTV virion by using a buffer consisting of high concentration of cations buffered at pH 5.5. The VP2 remained soluble in the buffer and was shown elicited a neutralising immune response in sheep (Huismans *et al.*, 1987a). With this method, VP2, in its authentic conformation, is removed from the virion and the VP2 remained soluble in the buffer. It was investigated if this buffer could be used in the de-aggregation of the insoluble aggregated VP2 expressed in Sf-9 insect cells and harvested 42 h after infection. Results are summarised in Table 2.2. The high salt concentration and low pH had no visible effect VP2 solubility. No difference in VP2 solubility was observed after incubation of the protein at low pH and high salt concentrations. Conditions for the removal of VP2 protein form the BTV virion were therefore not suitable for the de-aggregation of VP2. More drastic measures of solubilisation of VP2 were subsequently investigated.

2.3.4 Denaturing insoluble recombinant AHSV-3 VP2 with Guanidinium HCl and Urea

Inclusion bodies or insoluble aggregates are sometimes formed when recombinant proteins are expressed at very high concentrations in cells. Often these protein aggregates can be de-aggregated by chemical methods and then refolded to improve the solubility of the proteins. An attempt was made to de-aggregate VP2 by solubilisation with the use of different organic detergents in combination with β-Mercapto Ethanol as reducing agent. Sf-9 insect cells infected with AHSV-3 VP2 specific recombinant baculoviruses were harvested at 72 hpi when most of the VP2 protein was shown to be in an aggregated form. The cells were lysed in a buffer containing 1XPBS, 0.1% Triton X-100, 40μg/ ml DNAse and 20μg/ml RNAse followed by mechanical lysis through a .22 gauge needle. The aggregated VP2 was collected by centrifugation at 2000 rpm in a bench top centrifuge and then incubated for 2 hours at different temperatures with solubilisation buffers containing 8M UREA or 6M Guanidinium HCl with or without β-Mercapto Ethanol. Table 2.3. summarises the results of these experiments.

Table 2.2: A summary of AHSV-3 VP2 solubilisation with low pH and high salt concentration. The + indicate the relative amounts of VP2 protein found in the 2000 rpm pellet fraction (Column 2), the 10000 rpm pellet fraction (Column 3) and the 10 000 rpm supernatant fraction (Column 4). Row 2 represents the fractions of the AHSV-3 VP2 control experiment where the cells insect were lysed and incubated in 1X STE buffer. Row 3 represents the experiment where the insect cells expressing recombinant AHSV-3 VP2 were lysed and subsequently incubated in a buffer with high salt concentration at pH 5.5.

FRACTION	2000 rpm PELLET	10 000 rpm PELLET	10 000 rpm SUPERNATANT
Lysed cells by 1X PBS 0.1% Triton X	+++	-	+
Lysed cells solubilisation buffer (MES pH 5.5; 0.5M $MgCl_2$)	+++	-	+

Table 2.3: The deaggregation of AHSV-3 VP2 denaturing by means of different denaturing methods. The + indicates the relative amounts of VP2 .

Denaturing Buffer	Incubation temperature	Soluble protein	Aggregated protein
6M Guanidinium HCl	4°C	++	+
	20°C	+++	
	37°C	+++	
	50°C	+++	
6M Guanidinium HCl / β-Mercapto Ethanol	4°C	++	+
	20°C	++	+
	37°C	+++	
8M Urea	4°C	++	+
	20°C	++	+
	37°C	++	+
8M Urea / β-Mercapto Ethanol	4°C	++	+
	20°C	++	+
	37°C	++	+

Guanidinium HCl (6M) with or without β- Mercapto Ethanol resulted in the successful de-aggregation VP2. At 4°C the solubilisation was incomplete but at higher temperatures all of the VP2 was solubilised. Urea (8M) was less effective. Only partial VP2 de-aggregation was obtained under the various conditions applied. De-aggregation with Guanidinium HCl was therefore used for further experiments.

2.3.5 Refolding of the denatured AHSV-3 VP2

If the de-aggregated AHSV-3 VP2 were to be used as a subunit vaccine it would depend on the effective removal of the highly toxic Guanidinium HCl. To establish if the VP2 protein would remain soluble at low concentrations of Guanidinium HCl, the Guanidinium HCl was slowly diluted with 1X PBS to a final concentration of 0.75 M. It was found that at these concentrations VP2 remained soluble. An attempt was then made to remove the Guanidinium HCl from the solubilised protein as effectively as possible by size exclusion chromatography.

The G75 Sephadex columns were first equilibrated with BSA in 1XPBS and with Guanidinium HCl buffer to determine the column exclusion volume. The de-aggregated VP2 protein in 6M Guanidinium HCl (Figure 2.2 (a)) was diluted to 3M Guanidinium HCl with 1X PBS and fractionated on the size exclusion column. The fractions containing protein were determined by spectrophotometry, pooled and then used for further analysis.

To determine if size exclusion chromatography was effective for the desalting of VP2, the amount and the solubility of VP2 pooled was assessed. Half of the de-salted, pooled protein was freeze dried to concentrate the protein for quantitative analysis by SDS PAGE electrophoresis (Figure 2.2 (b)). The rest of the sample was centrifuged at 10000g for 1h in the SW 50.1 rotor of the Beckman ULTRA centrifuge to confirm that the protein passed through the column was soluble. The pellet was resuspended in 1XPBS and the supernatant was concentrated for analytical purpose by freeze drying. The samples were analysed by SDS PAGE gel electrophoresis.

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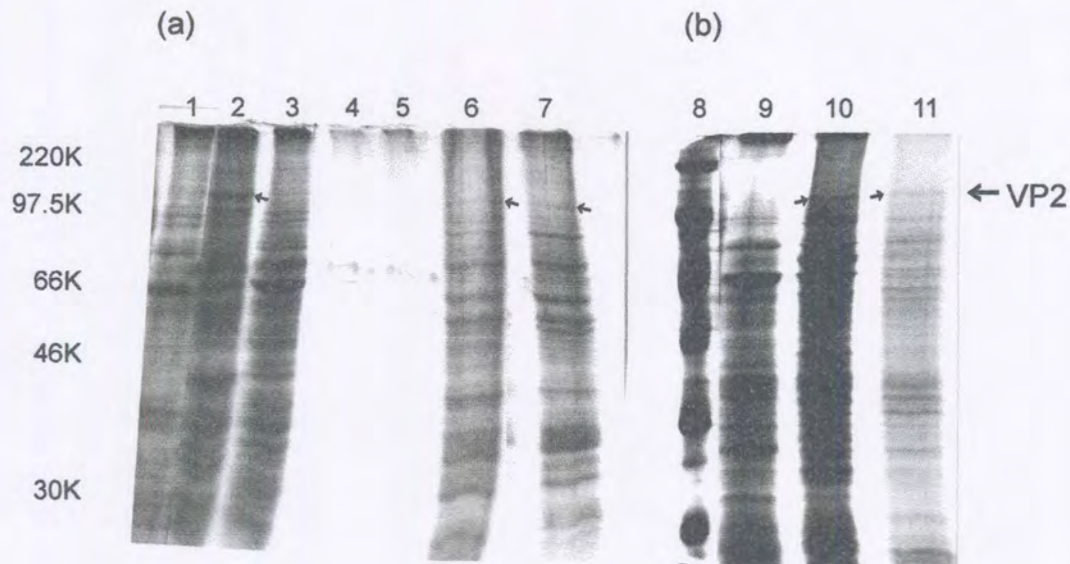

Figure 2.2 (a) and (b) : SDS PAGE gel analysis of de-aggregated and refolded proteins of AHSV-3 VP2 recombinant baculovirus infected insect cells harvested 72 hpi. Figure **(a)** presents proteins de-aggregated with 6 M Guanidinium HCl followed by de-salting with G75 Sephadex column. Figure **(b)** presents the soluble fraction of the de-salted proteins after centrifugation of the proteins at 10 000 rpm for 1h.

Lane 1: Wild type infected insect cells
Lane 2: 2000 rpm pellet of lysed AHSV-3 VP2 infected insect cells
Lane 3: 2000 rpm supernatant of AHSV-3 VP2 infected insect cells
Lane 4: 2000 rpm pellet of de-aggregated proteins
Lane 5: 10 000g pellet of de-aggregated proteins
Lane 6: 10 000g supernatant of AHSV-3 VP2 de-aggregated with GHCl
Lane 7: Total fraction of desalted AHSV-3 VP2
Lane 8: Molecular weight marker
Lane 9: Wild type infected insect cells
Lane 10: 10 000g supernatant of AHSV-3 VP2 de-aggregated by GHCl
Lane 11: Soluble fraction of AHSV-3 VP2 after desalting on G75 Sephadex column followed by centrifugation at 10 000g for 1h.

The results of the de-aggregation and de-salting process are presented in Figure 2.2 (a) and (b). Lanes 1- 3 illustrate the aggregated form in which VP2 is found in Sf-9 cells when harvested at 72hpi. The deaggregation of VP2 is illustrated in lanes 4 to 6. No aggregated VP2 was present after Guanidinium HCl treatment all the proteins were found in lane 6 representing the soluble protein fraction. The total amount of protein collected from the Sephadex column is shown in lane 7. The quantitative analysis of VP2 separated by the column is illustrated in lanes 10 and 11. To quantify the amount of VP2 eluted from the Sephadex column twice the amount of soluble protein loaded in lane 6 was loaded in lane 10 and half of the total protein eluted from the column was freeze dried and loaded in lane 11. Originally 100X the amount of protein represented in lane 10 was loaded onto the Sephadex column. Therefore the amount of protein expected to be seen in lane 11 would be 50 X the amount seen in lane 10. However by visual comparison of VP2 protein concentration in lanes 10 and 11 a loss of 98% VP2 was estimated.

2.4 Discussion

VP2 is the primary serotype specific and neutralising antigen of the AHS virion (Roy *et al.*, 1994b) and is therefore a candidate for a subunit vaccine against AHSV infection (Burrage *et al.*, 1993). The recombinant baculovirus expressed AHSV-4 VP2 protein has been shown to elicit a neutralising serotype specific immune response in horses. This resulted in protection against homologous AHSV-4 challenge (Roy *et al.*, 1996, Stone-Marschat *et al.*, 1996). The AHSV-3 VP2 protein expressed by means of the baculovirus system has been shown to elicit neutralising antibodies in rabbits (Vreede & Huismans, 1994). Investigations on recombinant baculovirus expressed AHSV-5 VP2 have shown that only the soluble and not the aggregated AHSV-5 VP2 elicits neutralising immune response in horses (Melinda du Plessis; personal communication). Only the soluble baculovirus expressed VP2 protein of AHSV-4 were used in order to elicit a protective immune response in horses (Roy *et al.*, 1996). These results are in accordance with reports that solubilised VP2 of BTV elicited protective immune response in sheep (Huismans *et al.*, 1987a).

Neutralising epitopes of VP2 seem to be conformational dependent. This was illustrated by an in depth investigation in neutralising epitopes presented by baculovirus expressed AHSV-4 VP2 protein. Thirty VP2 specific monoclonal antibodies were used for the identification of neutralising epitopes. Eleven of the thirty monoclonal antibodies showed neutralising activity. VP2 was not recognised by most of these neutralising monoclonal antibodies. Non-linear epitopes were however recognised on VP2, but only with monoclonal antibodies containing no neutralising activity (Martinez-Torrecuadrada *et al.*, 1996). These results indicate that the baculovirus expressed VP2 does not necessarily optimally present neutralising epitopes.

It was determined that the AHSV-3 VP2 expressed in insect cells occurred in an aggregated form. We were therefore interested to establish conditions under which VP2 was found in a soluble form or to determine conditions under which VP2 could be solubilised. A small amount of soluble VP2 was still present at 36 hpi but the amount progressively decreased with the increase in VP2 concentration in the insect cells until at 72hpi no soluble VP2 could be detected. This experiment demonstrated that aggregation of proteins may be the result of accumulation of VP2 at high concentrations in the cell. It is known that high levels of protein expressed in heterologous cell systems often result in aggregation of the proteins (Jaenicke & Seckler, 1997, Neugebauer, 1990, Roy & Jones, 1996, Rudolph & Lilie, 1996). Aggregation is a cooperative process and it is often more advantageous for proteins to aggregate when present in high concentrations in a heterologous cell system (Jaenicke & Seckler, 1997). This advantage is possibly due to lower energy requirements for the cell (Neugebauer, 1990, Rudolph & Lilie, 1996).

Other factors such as drastic temperature changes and the presence of detergents may also play a part in protein aggregation (Franks, 1995). It was established that freeze thawing and high concentrations of detergents (>0.1%) resulted in increased VP2 aggregation. The best method to keep VP2 soluble during lysis was found to be the use a mechanical lysis methods. This was achieved with a dounce or syringe in combination with a lysis buffer containing 1X PBS and 0.1% Triton X100.

Lysis by freeze-thawing of the cells resulted in aggregation of the of the soluble AHSV-3 VP2. The VP2 protein structure seems sensitive to freezing. Franks (1995) states that freezing is the removal of water (as ice) and is therefore accompanied by dramatic increase in concentration of water soluble substances in the residual liquid phase. Soluble VP2 present in the cell may therefore become even more concentrated as a result of the freezing process and therefore aggregate.

Salt concentration and pH also play an important role in protein aggregation and de- aggregation (Franks, 1995, Hjelmeland & Chrambach, 1981). We have established that the majority of AHSV-3 VP2 protein occurs in an aggregated form in baculovirus infected insect cells. The effect of low pH (5.5) and high cationic concentration on the aggregated protein was investigated. These conditions were used to remove authentic VP2 from purified bluetongue virions resulting in soluble immunogenic VP2 (Huismans *et al.*, 1987a). No significant effect was however observed on the aggregated state of the VP2 proteins. Low pH and high cationic salt concentrations were therefore not suitable to solubilise the aggregated recombinant VP2 protein. This is almost certainly due to the fact that the aggregated protein in no way resembles the VP2 conformation as found on the virion. Therefore the cations could not interact effectively with the protein resulting in solubilisation by de-aggregation.

Aggregated proteins can be de-aggregated by solubilisation with detergents acting as solvents for highly hydrophobic proteins. Many detergents are available for protein de-aggregation but no specific guidelines are available to determine which detergents are best. Strong chaotrophs such as Guanidinium HCl and Urea are recommended as a starting point for protein de-aggregation experiments (Jaenicke & Seckler, 1997, Neugebauer, 1990, Rudolph & Lilie, 1996). An attempt was made to de-aggregate AHSV-3 VP2 with both the Urea and Guanidinium HCl as detergents. More consistent results were achieved with Guanidinium HCl than with Urea. The best results were obtained at 37°C after a two hour incubation period. The effects of reducing agents were also investigated but it did not seem to play an important role in VP2 aggregation.

To refold the protein in the absence of the detergent, size exclusion chromatography was used (Determann, 1969, Neugebauer, 1990, Rudolph & Lilie, 1996). The size exclusion chromatography is a slow method of desalting proteins. The soluble proteins move through the column and are separated from the proteins that precipitate on the column. This shifts the equilibrium of the protein folding process away from the formation of aggregates which requires less energy (Neugebauer, 1990, Rudolph & Lilie, 1996). The refolding process of AHSV-3 VP2 was however very inefficient. Most of the protein appeared to have precipitated onto the column with only a small amount of protein passing through the column. This process is also very time consuming, expensive and the results were unsatisfactory.

Other methods of de-aggregation using any of the many available detergents (Neugebauer, 1990) could still have been investigated. Refolding by means of desalting with dialysis or dilution (Neugebauer, 1990, Rudolph & Lilie, 1996) followed by the concentration of proteins with PEG is an alternative to size exclusion chromatography. However the cost and in particular the labor intensive nature of such a de-aggregation and refolding process makes a chemical approach impractical for development of a subunit vaccine. It is essential that the preparation procedure for a vaccine is as easy, effective and inexpensive as possible. An alternative biological approach in which the two proteins outer capsid proteins VP2 and VP5 are co-expressed will be investigated instead of other chemical processes with the aim of providing an easier more effective method to produce immunogenic AHSV-3 VP2 .

CHAPTER 3

The Characterisation of AHSV-3 VP5 gene.

3.1 Introduction

VP5 and VP2 are the major capsid proteins of the AHS virion. VP2 of orbiviruses has been shown to be the most variable of the viral proteins (Roy & Sutton, 1998). The VP5 protein on the other hand seems to be highly conserved amongst the BTV serotypes (Gould & Pritchard, 1988, Loudon *et al.*, 1991, Oldfield *et al.*, 1991). Indications are that the VP5 protein of AHSV serotypes is also highly conserved (du Plessis & Nel, 1997, Williams *et al.*, 1998). Apart from its structural importance, the specific function of the VP5 protein is unknown. Some involvement of AHSV VP5 in serotype specificity (Bremer *et al.*, 1990, Mertens *et al.*, 1989) and virulence (O'Hara *et al.*, 1998) has been suggested. Very little is known about the immunogenic characteristics of the VP5 structure and even less about the possibility of some form of protein-protein interaction of the two outer capsid proteins VP2 and VP5. Such interaction could enhance the solubility of VP2 and as such increase its immunogenicity. An indication for this was given by the fact that in conjunction with the AHSV-4 VP2 protein the AHSV-4 VP5 protein has been shown to enhance protective immune response against homologous virus challenge (Martinez-Torrecuadrada *et al.*, 1996).

However, to gain a better understanding about VP5 structure and its possible function it is necessary to gain more information about the variability in AHSV VP5 sequence amongst different AHSV serotypes. From this information the identification of possible serotype specific epitopes may lead to a better understanding of the regions of VP5 that are exposed to the surface of the virion or interact with other virus proteins.

Two of the nine AHSV serotypes had already been sequenced when this study was initiated (du Plessis & Nel, 1997, Iwata *et al.*, 1992b, Sakamoto *et al.*, 1994) a third

sequence became available much later (Williams *et al.*, 1998) indicating high conservation between serotypes 4 and 9 (du Plessis & Nel, 1997) and serotypes 4 and 6 (Williams *et al.*, 1998) of the AHSV VP5 protein sequence.

In this chapter the nucleotide and amino acid sequences of AHSV-3 VP5 gene are described for the first time and compared to the VP5 nucleotide and amino acid sequences of the other AHSV serotypes. Highly variable regions were identified and correlated to predicted AHSV-3 VP5 secondary structure.

3.2 Materials and Methods

3.2.1 Double stranded DNA isolation and purification

Alkaline lysis method of Brinboim and Doly (1979) as described by Sambrook *et al.* (1989) and Ausubel *et al.* (1988) was used for plasmid extraction from liquid cultures of bacterial cells.

A single bacterial colony was transferred, with a sterile toothpick, to 3-5ml of LB medium (Luria-Bertani medium), containing the appropriate antibiotics and incubated overnight at 36°C with agitation. Three milliliters of the cell culture was harvested by centrifugation in a benchtop centrifuge at maximum speed for 1 minute. The medium was discarded and the harvested cells resuspended in 100µl of ice cold solution containing 25mM Tris-HCl pH 8.0, 50mM glucose and 10mM EDTA. After 5 minutes incubation period on ice, 200µl of freshly prepared 0.2N NaOH and 1% SDS was added to the cells, mixed gently and incubated ice for 5 minutes. Thereafter sodium acetate (150µl 3M NaAc) was added and mixed thoroughly. After incubation on ice for 10 minutes the plasmids in the supernatant were collected by centrifugation at maximum speed in a bench centrifuge for 10 minutes. The supematant was transferred into a clean tube and ethanol precipitated. The isolation was scaled up as required. Isolated plasmid was characterized together with appropriate DNA controls on a 1% agarose gel, containing Ethidium Bromide (0.5µg/ml) for DNA visualization under UV light, and electrophoresed in buffer (0.04M Tris acetate; 1mM EDTA pH 8.5). Restriction endonucleases (RE) (3.2.3) were also used for the characterisation of the recombinant plasmid. To obtain plasmid free from protein contamination the DNA was purified using the phenol/chloroform method (3.2.2). To obtain plasmid free form RNA, protein and contaminating salt, Nucleobond® (Nucleobond manual) and Quiagen™ (3.2.12) kits for plasmid isolation were used.

3.2.2 Phenol/chloroform extraction

Protein contamination was removed from extracted plasmid DNA (3.2.1) by the phenol/chloroform method (Sambrook *et al.*, 1989). The plasmid volume was made up to 400µl with TE buffer (10mM Tris pH 7.4 ; 1mM EDTA pH 8). Equal volumes of phenol : chloroform (in a 1:1 ratio of phenol : chloroform) were added. The sample was mixed until an emulsion formed. The mixture was centrifuged for 5 minutes at top speed in a bench centrifuge to separate the organic and aqueous phases. The aqueous phase containing the plasmid DNA was transferred into a new tube and the organic phase discarded. Residual phenol was removed by repeated chloroform extraction of the aqueous phase. Finally the DNA was precipitated out of the aqueous phase with in 0.3M Sodium Acetate (final concentration) with 2.5 volumes of 96% ethanol.

3.2.3 Restriction enzyme digestion

Restriction enzyme (RE) digests of plasmid DNA were carried out in the recommended salt buffer supplied with the enzyme (Promega; Boehringer Mannheim) for 1 hour at the recommended

temperature. Digestion products were analysed on 1% agarose gels using both undigested plasmid DNA and DNA markers of known molecular weight as controls.

3.2.4 Vector dephosphorilation

Dephosphorilation of linearised plasmid DNA was carried out by diluting the RE digest reaction 4X with a $^{1}/_{10}$ of 10X dephosphorilation buffer (0.5M Tris-HCl; 1mM EDTA pH 8.5), water and the addition of 1U calf intestine alkaline phosphotase (Boehringer Mannheim). The reaction was incubated at 37°C for 20 minutes. The enzyme was denatured at 94°C for 5 minutes and the DNA cooled down slowly to RT. The DNA was analysed by 1% agarose gel electrophoresis to ensure complete digestion. The band of interest was excised from the gel and purified by Geneclean^{TM} III DNA purification method (3.2.6).

3.2.5 Klenow

Linearised plasmid or digestion products were blunt ended by diluting the RE digestion reaction twice with $^{1}/_{10}$ buffer (10mM Tris HCl pH 8.0; 5mM $MgCl_2$; 100mM NaCl; 1mM 2- mercapto ethanol), 5mM dNTP's and water. Two units of Klenow enzyme (Boehringer Mannheim) modified DNA polymerase with a 5'-3' exonuclease and 3'-5' polymerase action was added. The reaction was incubated at 37°C for 30 minutes. The Klenow enzyme was heat inactivated before the DNA was analysed on a gel and the relevant fragments purified using Glassmilk^{TM} DNA purification (3.2.6).

3.2.6 Geneclean^{TM} III purification of DNA

Recombinant plasmids were constructed by cloning DNA fragments of interest, prepared by RE digestion, into linearised (3.2.3), dephosphorilated vector (3.2.4) blunt ended by Klenow (3.2.6) if required. The DNA was separated by 1% agarose gel electrophoresis. The fragments of interest were cut out of the gel and purified through Geneclean^{TM} III kit (BIO-101). The agarose slice containing the fragment of interest was melted at 52°C in 2.5 volume of 6M NaI followed by the addition of 5-10μl of the glassmilk slurry provided in the Geneclean^{TM} III kit. The glassmilk is a specially formulated silica matrix which binds single stranded and double stranded DNA selectively. Binding of the DNA to the silica matrix was allowed by incubating at RT for 5' while agitating, followed by incubation on ice for at least 20 minutes. The glassmilk bound DNA was washed 3X with 0.5M NEW washing buffer (NaCl; Tris; EDTA; Ethanol; water). The DNA was eluted twice from the silica matrix in 10μl water at 52°C for 5 minutes.

3.2.7 DNA ligation

Restriction fragments of foreign DNA were ligated into linearised pBS vector (Stratagene) by 1U of T4 DNA ligase (Boeringer Mannheim) in a ligation mix composed of $^{1}/_{10}$ ligation buffer (66mM Tris-HCl; 5mM $MgCl_2$; 1mN DTT;1mM ATP; pH 7.5), a vector : insert ratio of 3:1 for overhang and 5:1 for blunt end ligation, and water in a final volume of 20μl. The mix was incubated at 16°C overnight before transformation (3.2.9) into competent cells (3.2.8).

3.2.8 Preparation of *E.coli* competent cells

Competent cells were prepared by the calcium chloride method, of Cohen *et al.* as described by Sambrook *et al.* (1989) whereby calcium ions enable cells to take up DNA. *E.coli* HB101 or XL-1 blue cells (Sambrook *et al.*, 1989) were prepared routinely using this method for the transformation of competent cells with recombinant plasmid. The *E. coli* HB101 cells were maintained on agar plates containing no antibiotics and XL-1 blue cells on agar plates containing tetracycline hydrochloride (12.5μg/ml). A single colony of *E. coli* HB101 cells was inoculated into 3ml LB medium containing no antibiotics or a single colony of XL-1 blue cells was inoculated into 3ml LB medium containing tetracycline hydrochloride (12.5μg/ml) and grown at 37°C overnight with agitation. One milliliter of the cell culture was inoculated into 100ml of medium containing the relevant antibiotics and grown at 37°C until the logarithmic (log) phase (OD_{550} = 0.5) was reached. The cells were harvested by centrifugation in a pre-chilled sterile tube at 2000g for 5 minutes at 4°C. The collected cells were resuspended in half of the original volume of ice cold freshly prepared sterile 50mM $CaCl_2$ (calcium chloride) and incubated on ice for 15 minutes. The cells were collected again by centrifugation and resuspended in $^{1}/_{20}$ original

volume $CaCl_2$. The cells were incubated another hour before use. Remaining competent cells were snap frozen in 15% glycerol and stored at -70°C for up to three months for further use.

3.2.9 Transformation of competent cells with plasmid DNA

Plasmids were transformed into competent cells (3.2.8) using the heat shock method. Plasmid DNA was added to 100µl of appropriate competent cells. The DNA was allowed to adsorb to the cells for 30 minutes on ice followed by a 90 second heat shock at 42°C. Thereafter the cells were cooled in ice for 2 minutes. LB medium (900µl) was added to the cells. After an incubation period of 1 hour at 37°C with agitation. Cell aliquots of 100µl were plated out with 50µl of 2% 5-Bromo-4-chloro-3-indonyl-βD galactopyranoside (X-gal) in dimethylformamide (DMF) and 10µl 100mM isopropyl-β-D thiogalactopyranoside (IPTG) on agar plates (1.2% agar in LB medium) containing tetracycline hydrochloride (12.5µg/ml) and ampicillin (100µl/ml) for XL-1 blue cells. Plates were incubated overnight at 37°C. Colonies of desired phenotype were picked with sterile toothpicks and grown up in 3-5 ml LB medium with appropriate antibiotics for further selection of recombinant plasmid.

3.2.10 Selection of recombinant plasmid.

Recombinant plasmids were selected by plasmid isolation form white colonies picked up and grown in 5 ml of culture. The isolated plasmids (3.2.1) were analysed by 1% agarose gel electrophoresis with a non-recombinant plasmids as control. Recombinant plasmids were selected and confirmed by restriction enzyme digests (3.2.3) and analysis on a 1% agarose gel.

3.2.11 Subcloning of AHSV-3 VP5 gene

A cDNA clone of AHSV-3 VP5 cloned into pBR322 vector was kindly provided by Frank Vreede. A subclone of approximately 400 bp representing the 5' end of the gene was generated by restriction with Bgl II and Bam HI (3.2.3) and cloned into the Bam HI site of the pBS vector (Stratagene). The 3' end (+/- 350 bp) was subcloned by restriction with Hind III and cloned into the Hind III site of the pBS vector. The central section of the gene consisting of approximately 800bp was restricted with Hind III and Bgl II and cloned into the Bam HI and Hind III sites of the pBS vector.

3.2.12 Plasmid isolation for Automated sequencing

A Quiagen plasmid isolation kit was used for plasmid isolation prepared for automated sequencing. This method is based on alkaline lysis followed by purification of the DNA by Anion-Exchange Quiagen® column following standard recommended protocol (The buffers used were provided by the suppliers and are indicated according to suppliers names and buffer components.). Cells from a 5ml bacteria culture were harvested by centrifugation and the supernatant discarded. The cells were resuspended in ice cold buffer P1 (50mM Tris HCl pH 8.0; 10mM EDTA; 100µg/ml RNAse A). Buffer P2 (200mM NaOH; 1% SDS) was added to the cell suspension and incubated for 5 minutes followed by the addition of buffer P3 (3.0M KAc pH 5.5) and incubation on ice for 5 minutes. The precipitate of proteins, cell debris and chromosomal DNA was separated form the plasmid DNA by centrifugation for 10 minutes at top speed in a microfuge. The plasmid containing supernatant was placed on Quiagen® anion exchange resin tip-20 equilibrated with buffer QBT (750mM NaCl; 50mM MOPS pH 7.0; 15% isopropanol; 0.15% Triton X-100) and allowed to flow through the tip allowing the plasmid DNA to bind to the resin. The DNA was washed with buffer QC (10M NaCl; 50mM MOPS pH 7.0; 15% isopropanol) to remove all contaminants. The DNA was eluted form the resin by buffer QF (1.26M NaCl; 50mM Tris HCl, pH 8.5 ; 15% isopropanol) and concentrated by precipitation with isopropanol at room temperature to reduce precipitation of salt with the DNA. The purity and concentration of the DNA was determined by spectrophotometry at A_{320}, A_{260} and A_{280} as well as 1% agarose gel electrophoresis.

3.2.13 Cycle sequencing

Cycle sequencing for automated sequencing analysis is based on the Sanger dideoxy mediated chain termination method. Very high quality DNA (200µg-500µg) (3.2.12) was mixed with 3,2 pmol of primer (M13 forward or M13 reverse primer), water and 8.0µl of the Big Dye™ Terminal Ready reaction mix (Perkin Elmer) to a final volume of 20µl. Half (10µl) and quarter (5µl) reactions using 200µg of DNA and 3,2 pmol primer were also used. The reaction mix contains premixed dye terminators, deoxynucleoside

triphosphates, enzyme, $MgCl_2$ and buffer (Tris HCl pH 8.0). The AmpliTaq® enzyme is genetically modified to decrease discrimination against dideoxynucleotides and the 5'-3' nuclease activity is eliminated. The Dye terminators of the Big Dye™ kit are linked to dichlororhodamine (dRhodamine) acceptor dye. The mixed reaction was placed in a Perkin Elmer Gene Amp 9600 PCR system thermocycler for a 25 cycle of 96° thermal rapid ramp, 96°C 10 seconds DNA denaturing, 50°C, 10 second primer annealing and 60°C 4 minutes strand elongation. The DNA fragments were concentrated by ethanol precipitation (99.9% absolute Ethanol SARCHEM) and concentrated by 10 minute centrifugation in a bench centrifuge and air dried on the bench. The DNA was resuspended in loading buffer (1 [50mg/ml pH 8.0 Dextran blue: 25mM EDTA] : 5 [100% Deionized Formamide]) for sequence determination by ABI Prism® 377 automated sequencer. The data was extracted using Perkin Elmer software Sequence Analysis™ and processed using Sequence Navigator™ and Strider™ programs.

3.3 Results:

All methods used to obtain the following results were described in section 3.2. unless otherwise stated.

3.3.1 Subcloning of AHSV-3 VP5 cDNA

To determine the nucleotide sequence of the AHSV-3 VP5 gene a restriction enzyme map had to be determined for subcloning of the gene. An AHSV-3 VP5 cDNA clone, generated by reverse transcription of denatured dsRNA, was sequenced. The cDNA, cloned by G-C tailing into the Pst1 site of the pBR322 vector, was kindly provided by Frank Vreede. The 5' and 3' ends of the gene had been sequenced by Frank Vreede. For the determination of the complete sequence the gene was excised from the pBR322 vector with Pst1 and cloned into the Pst1 site of the pBS vector to enable subcloning and sequencing of the gene. A restriction map was determined and two unique sites, Bgl II (+/- position 400 bp) and Hind III (+/- position 1300) were used for the subcloning of the gene. The individual subclones were made and are illustrated in Figure 3.1, lanes 4, 5 and 6 and Figure 3.2. The subclones were excised form the pBS vector presenting the fragments of approximately 400 bp (lane 4), 800bp (lane 5) and 350 bp (lane 6). The full-length gene of 1566bp excised with PstI form pBS can be seen in lane 3. The digestion was however incomplete and therefore the linear and supercoiled forms, represented by the two top bands, can also bee seen in this lane. The third band corresponds with the linearized pBS (lane 2) and represents pBS from which the gene has been excised. These results confirm successful subcloning of the AHSV-3 VP5 gene.

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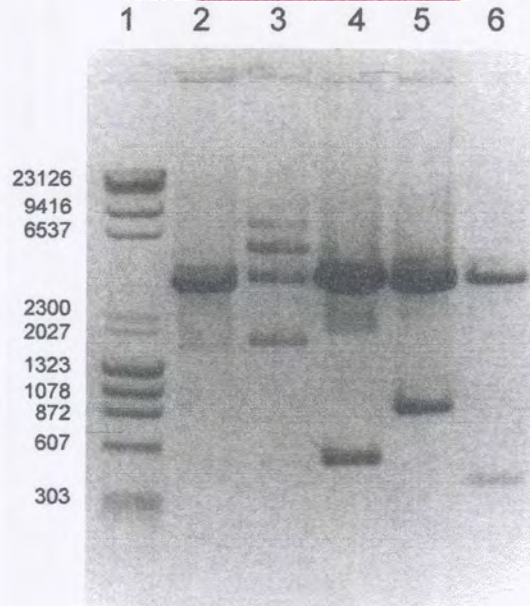

Figure 3.1: Restriction enzyme analysis of the VP5 gene of AHSV-3 on a 1% agarose gel. The subclones were digested with restriction enzymes as indicted below.
Lane 1: Molecular weight marker
Lane 2: Linearized pBS
Lane 3: AHSV-3 VP5 cDNA in pBS digested by PstI
Lane 4: Bam HI- Bgl II subclone in pBS digested by Bam HI and Hind III
Lane 5: Hind III- Bgl II subclone in pBS digested by Hind III and KpnI
Lane 6: Hind III- Hind III subclone in pBS digested by Hind III

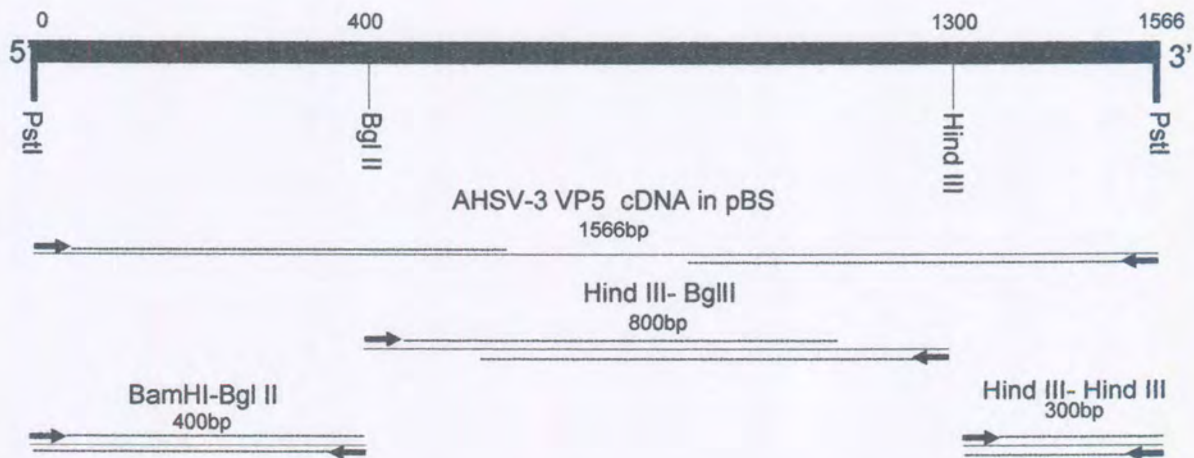

Figure 3.2: Restriction enzyme map of AHSV-3 VP5 gene and sequence strategy. The arrows indicate the direction in which the clones were sequenced and the perforated lines the approximate length of sequence obtained. The subclones are named according the restriction enzyme sites with which they were cloned. The approximate length of each subclone is indicated. Both strands strand of each subclone and the ends of the full-length cDNA were sequenced.

3.3.2 AHSV-3 VP5 nucleotide and amino acid sequence

The nucleotide sequence of AHSV-3 VP5 cDNA gene was determined by sequencing three subclones produced above as well as the 3' and 5' ends of the AHSV-3 VP5 cDNA clone in pBS (Figure. 3.1 and Figure. 3.2). M13 forward and reverse primers were used for sequencing. Sequences up to 800bp in length were determined enabling the sequencing of overlapping regions as indicated in Figure 3.2. The gene was found to be 1566 nucleotides in length. A 19 nucleotide 5' and a 29 nucleotide 3' non-coding region was identified flanking the coding region of the gene. The 5' and 3' termini of the AHSV-3 VP5 gene were determined to have the following sequences 5' GUUUAU 3' and 5'ACAUAC 3' respectively. The longest open reading frame of the AHSV-3 VP5 sequence was determined to stretch from the start codon AUG in position 20-22 to the stop codon TGA in position 1535-1537 (Figure. 3.3). The AUG is surrounded by the -3 A and the G^{4+}, the Kozak sequence described as the essential sequences for effective translation (Kozak, 1989, Kozak, 1991).

The amino acid sequence was deduced from the nucleotide sequence of AHSV-3 VP5 gene. A peptide consisting of 505 amino acids was translated composing a protein of 56.94 K with a pI of 6.30 (Figure 3.3). Kyte and Doolittle hydrophilicity values were used to determine the percentage hydrophilicity of the protein as 53% and the hydrophobicity as 46%. A Hydrophobicity plot, using these Kyte and Doolittle (1982) values is shown in Figure 3.4. The region above the center or zero line shows regions with hydrophobic amino acid values while the region below the line indicate hydrophilic regions. From the hydrophobicity plot two large predominantly hydrophilic regions interspersed with smaller hydrophobic sections were identified.

```
          1          11         21         31         41         51         60
          |          |          |          |          |          |          |
VP5AHSV3  GTTTATTTTT CCAGAGACCA TGGGTAAGTT CACATCCTTT TTGAAGCGCG CGGGTAGCGC
VP5AHSV3                      M --G--K--F- -T--S--F-- L--K--R--A --G--S--A-
          61         71         81         91         101        111        120
          |          |          |          |          |          |          |
VP5AHSV3  GACCAAGAAA GCACTAACTT CAGATGCAGC TAAGAGGATG TATAAAATGG CTGGTAAAAC
VP5AHSV3  -T--K--K-- A--L--T--S --D--A--A- -K--R--M-- Y--K--M--A --G--K--T-
          121        131        141        151        161        171        180
          |          |          |          |          |          |          |
VP5AHSV3  GTTACAGAAG GTCGTAGATA GTGAAGTGGG AAGTGCGGCG ATAGATGGAG TAATGCAAGG
VP5AHSV3  -L--Q--K-- V--V--D--S --E--V--G- -S--A--A-- I--D--G--V --M--Q--G-
          181        191        201        211        221        231        240
          |          |          |          |          |          |          |
VP5AHSV3  AACAATTCAG AGCATAATAC AGGGTGAAAA TTTGGGAGAT TCAATCAAAC AAGCAGTAAT
VP5AHSV3  -T--I--Q-- S--I--I--Q --G--E--N- -L--G--D-- S--I--K--Q --A--V--I-
          241        251        261        271        281        291        300
          |          |          |          |          |          |          |
VP5AHSV3  TTTGAATGTA GCTGGCACGT TAGAGTCGCC TCCAGATCCA TTAAGTCCAG GTGAACAACT
VP5AHSV3  -L--N--V-- A--G--T--L --E--S--P- -P--D--P-- L--S--P--G --E--Q--L-
          301        311        321        331        341        351        360
          |          |          |          |          |          |          |
VP5AHSV3  ATTATATAAT AAAGTATCTA AAATCGAAAG AGCGGAGAAG GAAGATCGCG TGATTGAGAC
VP5AHSV3  -L--Y--N-- K--V--S--K --I--E--R- -A--E--K-- E--D--R--V --I--E--T-
          361        371        381        391        401        411        420
          |          |          |          |          |          |          |
VP5AHSV3  GCATAATGAA AAGATTATTG AAAAGTATGG GGAAGATCTA TTGAAAATCC GGAAAATTAT
VP5AHSV3  -H--N--E-- K--I--I--E --K--Y--G- -E--D--L-- L--K--I--R --K--I--M-
          421        431        441        451        461        471        480
          |          |          |          |          |          |          |
VP5AHSV3  GAAGGGAGAG GCTGAAGCGG AGCAGCTCGA GGGAAAAGAA ATGGAATACG TCGAAAAAGC
VP5AHSV3  -K--G--E-- A--E--A--E --Q--L--E- -G--K--E-- M--E--Y--V --E--K--A-
          481        491        501        511        521        531        540
          |          |          |          |          |          |          |
VP5AHSV3  GCTAAAGGGC ATGCTCAAAA TCGGAAAAGA TCAATCTGAG CGTATCACAC GGTTGTATCG
VP5AHSV3  -L--K--G-- M--L--K--I --G--K--D- -Q--S--E-- R--I--T--R --L--Y--R-
          541        551        561        571        581        591        600
          |          |          |          |          |          |          |
VP5AHSV3  CGCCCTCCAA ACGGAAGAGG ATTTAAGAAC TTCGGATGAG ACGAGGATGA TCAGCGAGTA
VP5AHSV3  -A--L--Q-- T--E--E--D --L--R--T- -S--D--E-- T--R--M--I --S--E--Y-
          601        611        621        631        641        651        660
          |          |          |          |          |          |          |
VP5AHSV3  TAGAGAAAAA TTTGATGCGT TGAAACAGGC AATTGAACTT GAGCAGCAGG CAACGCATGA
VP5AHSV3  -R--E--K-- F--D--A--L --K--Q--A- -I--E--L-- E--Q--Q--A --T--H--E-
          661        671        681        691        701        711        720
          |          |          |          |          |          |          |
VP5AHSV3  GGAAGCTGTG CAGGAGATGT TGGATTTAAG TGCCGAGGTC ATCGAGACGG CGGCTGAAGA
VP5AHSV3  -E--A--V-- Q--E--M--L --D--L--S- -A--E--V-- I--E--T--A --A--E--D-
          721        731        741        751        761        771        780
          |          |          |          |          |          |          |
VP5AHSV3  TTTACCAATC TTTGGCGCTG GCGCTGCAAA TGTTGTTGCG ACGACACGCG CAATTCAAGG
VP5AHSV3  -L--P--I-- F--G--A--G --A--A--N- -V--V--A-- T--T--R--A --I--Q--G-

          781        791        801        811        821        831        840
          |          |          |          |          |          |          |
VP5AHSV3  GGGCCTGAAG TTGAAGGAGA TAATAGATAA ACTCACAGGG ATTGATCTCT CTCATTTAAA
VP5AHSV3  -G--L--K-- L--K--E--I --I--D--K- -L--T--G-- I--D--L--S --H--L--K-
          841        851        861        871        881        891        900
          |          |          |          |          |          |          |
VP5AHSV3  AGTAGCGGAT ATCCATCCTC ATATAATTGA GAAAGCTATG CTAAAGGATA AAATCCCAGA
VP5AHSV3  -V--A--D-- I--H--P--H --I--I--E- -K--A--M-- L--K--D--K --I--P--D-
          901        911        921        931        941        951        960
          |          |          |          |          |          |          |
VP5AHSV3  TAATGAGTTG GCGATGGCGA TAAAATCGAA GGTTGAGGTT ATTGATGAAA TGAATACGGA
VP5AHSV3  -N--E--L-- A--M--A--I --K--S--K- -V--E--V-- I--D--E--M --N--T--E-
          961        971        981        991        1001       1011       1020
          |          |          |          |          |          |          |
VP5AHSV3  GACAGAACAC GTTATTGAGT CCATCATGCC TCTAGTGAAG AAGGAATACG AAAAACATGA
VP5AHSV3  -T--E--H-- V--I--E--S --I--M--P- -L--V--K-- K--E--Y--E --K--H--D-
          1021       1031       1041       1051       1061       1071       1080
          |          |          |          |          |          |          |
VP5AHSV3  TAACAAATAC CATGTGAATA TACCAAGTGC GTTGAAAATA CATTCAGAAC ATACGCCAAA
VP5AHSV3  -N--K--Y-- H--V--N--I --P--S--A- -L--K--I-- H--S--E--H --T--P--K-
          1081       1091       1101       1111       1121       1131       1140
          |          |          |          |          |          |          |
VP5AHSV3  GGTACATATA TACACTACGC CGTGGGACTC TGATAAAGTT TTTATATGCA GATGCATTGC
VP5AHSV3  -V--H--I-- Y--T--T--P --W--D--S- -D--K--V-- F--I--C--R --C--I--A-
          1141       1151       1161       1171       1181       1191       1200
          |          |          |          |          |          |          |
VP5AHSV3  GCCGCATCAT CAGCAGAGGA GTTTTATGAT TGGATTTGAT TTAGAGATTG AATTCGTCTT
VP5AHSV3  -P--H--H-- Q--Q--R--S --F--M--I- -G--F--D-- L--E--I--E --F--V--F-
          1201       1211       1221       1231       1241       1251       1260
          |          |          |          |          |          |          |
VP5AHSV3  TTATGAGGAT ACCTCGGTTG AAGGTCACAT AATGCATGGG GGAGCGGTGT CGATCGAGGG
VP5AHSV3  -Y--E--D-- T--S--V--E --G--H--I- -M--H--G-- G--A--V--S --I--E--G-
          1261       1271       1281       1291       1301       1311       1320
          |          |          |          |          |          |          |
VP5AHSV3  ACGAGGATTT AGACAAGCTT ATAGTGAGTT CATGAACGCG GCTTGGTCTA TGCCCTCAAC
VP5AHSV3  -R--G--F-- R--Q--A--Y --S--E--F- -M--N--A-- A--W--S--M --P--S--T-
          1321       1331       1341       1351       1361       1371       1380
          |          |          |          |          |          |          |
VP5AHSV3  TCCAGAGCTG CATAAGAGAA GATTGCAACG TAGTTTAGGT TCACATCCGA TTTATATGGG
VP5AHSV3  -P--E--L-- H--K--R--R --L--Q--R- -S--L--G-- S--H--P--I --Y--M--G-
          1381       1391       1401       1411       1421       1431       1440
          |          |          |          |          |          |          |
VP5AHSV3  ATCGATGGAT TATACTGTAA GTTATGAACA GCTCGTTTCG AACGCGATGA AGTTAGTTTA
VP5AHSV3  -S--M--D-- Y--T--V--S --Y--E--Q- -L--V--S-- N--A--M--K --L--V--Y-
          1441       1451       1461       1471       1481       1491       1500
          |          |          |          |          |          |          |
VP5AHSV3  TGACACAGAC CTACAAATGC ACTGTTTACG TGGACCATTG AAATTCCAAA GGCGCACCCT
VP5AHSV3  -D--T--D-- L--Q--M--H --C--L--R- -G--P--L-- K--F--Q--R --R--T--L-
          1501       1511       1521       1531       1541       1551       1560
          |          |          |          |          |          |          |
VP5AHSV3  GATGAATGCG CTTCTCTTTG GTGTGAAAGT AGCTTGAAAG CCTCATGGCG CGGGAAAAAC
VP5AHSV3  -M--N--A-- L--L--F--G --V--K--V- -A--*-
          1561
          |
VP5AHSV3  ACATAC
VP5AHSV3
```

Figure 3.3: Complete nucleotide and deduced amino acid sequence of AHSV-3 VP5 gene. The termination codon is indicated by an *.

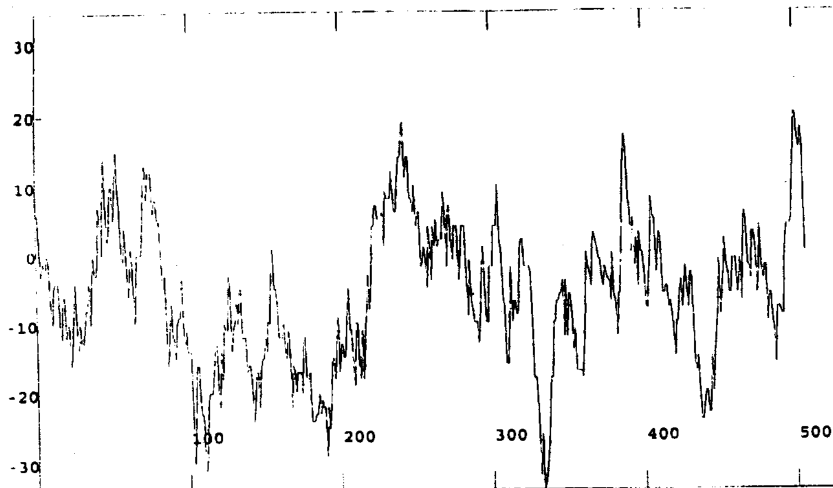

Figure 3.4: A hydrophobicity profile of AHSV-3 VP5 protein determined according to Kyte and Doolittle hydrophobicity values (Kyte & Doolittle, 1982). The regions above the center line indicate the hydrophobic areas and the sections below the center line indicate the hydrophilic regions of the AHSV-3 VP5 protein.

3.3.3 Comparative analysis of AHSV VP5 nucleotide sequences

The nucleotide sequence of AHSV-3 VP5 was further analysed by comparison to known AHSV VP5 nucleotide sequences using Clustal X alignment matrix (Higgins & Sharp, 1988). The comparative analysis was used to determine if the nucleotide sequence of AHSV-3 VP5 was conserved relative to the other known AHSV VP5 nucleotide sequences. Figure 3.5 is a representation of the nucleotide alignment of the different AHSV VP5 genes. The red regions represent 100% conservation, the blue regions 50% conservation and the white regions less than 50% conservation of the nucleotide sequence of the gene. The average conservation amongst different nucleotide sequences of the AHSV VP5 genes is summarised in Table 3.1.

Figure 3.5: Clustal X alignment of nucleotide sequence of AHSV-3 VP5, AHSV-6 VP5 (Williams *et al*, 1998), AHSV-9 VP5 (du Plessis, *et al* 1997, AHSV-4 VP5 (I) (Iwata *et al* ,1992) and AHSV-4 VP5 (S) vaccine strain (Sakomoto *et al*, 1994)genes. The red regions represent 100% conservation, the blue 50% conservation and the white less than 50% conservation of nucleotide sequence with respect to AHSV-3 VP5 nucleotide sequence.

Table 3.1: Conservation of nucleotide sequences of AHSV-3 VP5 and nucleotide sequences of AHSV-6 VP5 (Williams *et al.*, 1998), AHSV-9 VP5 (du Plessis & Nel, 1997)., AHSV-4 VP5 (I) (Iwata *et al.*, 1992b) and AHSV-4 VP5 (S) (Sakamoto *et al.*, 1994) genes.

	AHSV-3 VP5	AHSV-6 VP5	AHSV-9 VP5	AHSV-4 VP5 (I)	AHSV-4 VP5 (S)
AHSV-3 VP5		91%	93%	74%	74%
AHSV-6 VP5			88%	71%	71%
AHSV-9 VP5				74%	74%
AHSV-4 VP5 (I)					97%
AHSV-4 VP5 (S)					

3.3.4 Comparative analysis of AHSV VP5 amino acid sequences

The amino acid composition of AHSV-3 VP5 was compared to known AHSV VP5 (Table 3.2). Amino acid sequence alignments were used to identify potentially important regions on the VP5 protein. Clustal X alignment (Higgins & Sharp, 1988) was used to compare the amino acid sequence of AHSV-3 VP5 with known AHSV amino acid sequences (Figure 3.6). Predicted similarity and identity values resulting from the Clustal X alignment of cognate AHSV VP5 amino acid sequences are shown in Table 3.3. AHSV and BTV are closely related. Data generated from BTV VP5 amino acid alignments can therefore be used for comparison with AHSV VP5 data. For this purpose randomly selected BTV VP5 amino acid sequences were aligned (Figure 3.7) and thereafter AHSV VP5 amino acid sequences were aligned with BTV VP5 sequences (Figure 3.8) using Clustal X alignment matrix.

Table 3.2 : Comparison of amino acid composition of AHSV-3 VP5 with AHSV-6 VP5 (Williams *et al.*, 1998); AHSV-9 VP5 (du Plessis & Nel, 1997) AHSV-4 VP5 (I)(Iwata *et al.*, 1992b) and AHSV-4 VP5 (S) (Sakamoto *et al.*, 1994). The bold numbers represent the total of a specific amino acid present whole the numbers in brackets represent the percentage of a particular amino acid present.

Amino Acid	**AHSV-3 VP5**	**AHSV-6 VP5**	**AHSV-9 VP5**	**AHSV-4 VP5 (I)**	**AHSV-4 VP5 (S)**
Alanine (A)	**40** (7.92)	**37** (7.92)	**41** (8.11)	**43** (8.51)	**42** (8.31)
Cysteine (C)	**3** (0.59)	**3** (0.59)	**4** (0.79)	**2** (0.39)	**2** (0.39)
Aspartic acid (D)	**27** (5.46)	**24** (4.67)	**23** (4.55)	**29** (5.74)	**29** (5.94)
Glutamic acid (E)	**51** (10.09)	**54** (10,71)	**51** (10.090	**49** (9.7)	**49** (9.7)
Phenylalanine(F)	**13** (2.57)	**13** (2.57)	**14** (2.77)	**13** (2.570	**13** (2.57)
Glycine (G)	**30** (5.94)	**30** (5.94)	**32** (6.33)	**32** (6.33)	**32** (6.33)
Histidine (H)	**19** (3.56)	**18** (3.5)	**20** (3.96)	**19** (3.76)	**20** (3.96)
Isoleucine (I)	**40** (7.92)	**41** (8.11)	**41** (8.11)	**43** (8.51)	**43** (8.51)
Lysine (K)	**44** (8.71)	**40** (7.92)	**43** (8.51)	**40** (7.92)	**42** (8.31)
Leucine (L)	**43** (8.51)	**43** (8.51)	**38** (7.52)	**43** (8.51)	43 (8.51)
Methionine (M)	**22** (4.35)	**21** (4.16)	**22** (4.35)	**18** (3.57)	**18** (3.57)
Asparagine (N)	**12** (2.37)	**13** (2.57)	**13** (2.57)	**13** (2.57)	**12** (2.37)
Proline (P)	**16** (3.16)	**15** (2.97)	**16** (3.16)	**14** (2.77)	**15** (2.97)
Glutamine (Q)	**21** (4.15)	**22** (4.35)	**23** (4.55)	**21** (4.150	**21** (4.15)
Arganine (R)	**22** (4.35)	**24** (4.75)	**22** (4.35)	**24** (4.75)	**23** (4.55)
Serine S	**31** (6.13)	**32** (6.34)	**30** (5.95)	**26** (5.14)	**27** (5.35)
Threonine (T)	**26** (5.14)	**26** (5.14)	**26** (5.14)	**28** (5.54)	**28** (5.54)
Valine (V)	**29** (5.74)	**32** (6.34)	**31** (6.13)	**31** (6.13)	**31** (6.13)
Tryptophan (W)	**2** (0.39)	**1** (0.19)	**2** (0.39)	**2** (0.39)	**2** (0.39)
Tyrosine (Y)	**15** (2.97)	**15** (2.97)	**13** (2.57)	**15** (2.97)	**14** (2.77)
Total	505 (100)	504 (100)	505 (100)	505 (100)	505 (100)

Figure 3.6: Clustal X (Higgins & Sharp, 1988) amino acid alignment of AHSV-3 VP5, AHSV-6 VP5 (Williams *et al.*, 1998), AHSV-9 VP5(du Plessis & Nel, 1997), AHSV-4 VP5 (I) (Iwata *et al.*, 1992b) and AHSV-4 VP5 (S) (Sakamoto *et al.*, 1994). The red regions represent 100%, the blue 50% and the white less than 50% conservation.

Table 3.3: Comparison of AHSV-3 VP5 protein cognate AHSV VP5 proteins. Identity is indicated in **bold** and similarity is indicated in *italic*.

	AHSV-3 VP5	AHSV-6 VP5	AHSV-9 VP5	AHSV-4 VP5 (I)	AHSV-4 VP5 (S)
AHSV-3 VP5		**95%**	**90%**	**83%**	**83%**
AHSV-6 VP5	*98%*		**89%**	**82%**	**82%**
AHSV-9 VP6	*94%*	*93%*		**80%**	**80%**
AHSV-4 VP5 (I)	*95%*	*94%*	*89%*		**98%**
AHSV-4 VP5 (S)	*95%*	*94%*	*90%*	*99%*	

Figure 3.7: Clustal X (Higgins & Sharp, 1988) amino acid alignment of randomly selected BTV amino acids. BTV-1 VP5 AUS (Gould & Pritchard, 1988); BTV-2 VP5 AUS (Hirasawa & Roy, 1990) BTV-1 SA (Wade-Evans *et al.*, 1988); BTV-10 SUB VP5 (Purdy *et al.*, 1986); BTV-17 VP5 (Yang & Li, 1992); BTV-13 VP5 (Oldfield *et al.*, 1991). The red regions represent 100%, the blue 50% and the white less than 50% conservation.

Figure 3.8: Clustal X (Higgins & Sharp, 1988) amino acid alignment of AHSV and randomly selected BTV VP5 amino acid sequences AHSV-3 VP5, AHSV-6 VP (Williams *et al*., 1998), AHSV-9 VP5 (du Plessis & Nel, 1997), AHSV-4 VP5 (I) (Iwata *et al*., 1992b) and AHSV-4 VP5 (S) (Sakamoto *et al*., 1994). BTV-1 VP5 AUS (Gould & Pritchard, 1988); BTV-2 VP5 AUS (Hirasawa & Roy, 1990) BTV-1 SA (Wade-Evans *et al*., 1988); BTV-10 SUB VP5 (Purdy *et al*., 1986); BTV-17 VP5 (Yang & Li, 1992); BTV-13 VP5 (Oldfield *et al*., 1991). The red regions represent 100% conservation of amino acids and the white regions less than 100% amino acid conservation.

The identification of regions on VP5 protein that may play an immunogenic role in the protection against AHS virus infection or in interact with other virus proteins such as VP2 was attempted. For this purpose amino acid alignments were used to calculate the average amino acid conservation spanning blocks of 50 amino acids along the AHSV VP5 gene (Figure 3.9 (a) i). This information was correlated with the predicted simplified hydrophobicity plot of AHSV-3 VP5 protein (Figure 3.9 (a) ii) determined according to the Kyte and Doolittle hydrophobicity values (Kyte & Doolittle, 1982). The hydrophobic regions are situated above and the hydrophilic regions situated below the center line of the graph. An antigenicity plot of AHSV-3 VP5 based on the prediction of hydrophilic regions with potential for eliciting possible B cell antibody response (Hopp & Woods, 1983) was also determined and subsequently aligned with the hydrophobicity plot (Figure 3.9 (a) iii). Similarly BTV VP5 peptides were analysed using BTV-10 VP5 (Purdy *et al.*, 1986) as the basis for the predicted hydrophilicity (Kyte & Doolittle, 1982) and antigenicity (Hopp & Woods, 1983) plots (Figure 3.9 (b)). Four regions, in similar location to those predicted for the AHSV VP5 (Figure 3.9 (a)) were predicted to be of possible importance for BTV VP5 (Figure 3.9 (b)).

From the amino acid sequence the secondary structure of AHVS-3 VP5 was predicted using the GOR I method (Garner *et al.*, 1978) (Figure 3.10 (a)). To determine if the secondary structure predicted for AHSV-3 VP5 was unique it was compared to the secondary structure predicted for AHSV-6 VP5 (Williams *et al.*, 1998) (Figure 3.10 (b)). The VP5 protein from both serotypes seems to be divided into two major regions. A α-helix domain represented by the dark blue bars and β-sheet domain represented predominantly by yellow bars.

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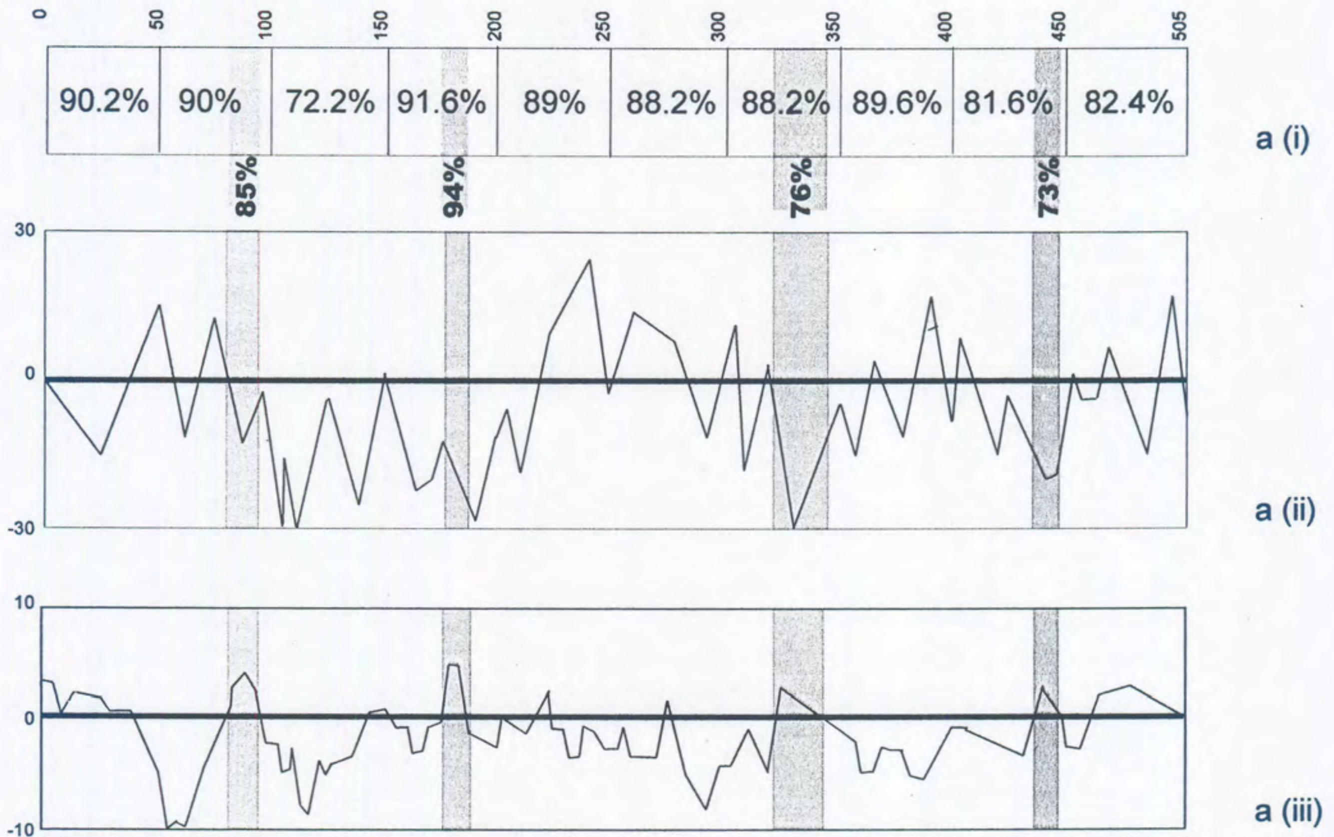

Figure 3.9 (a): Alignment of AHSV VP5 amino acid conservation profile determined by dividing the known AHSV VP5 amino acid sequences into blocks of 50 amino acid and calculating the average conservation of amino acids within the defined regions. (a(i)). A simplified hydrophobicity plot of AHSV-3 VP5 according to Kyte and Doolittle hydrophobicity values (1982) (a (ii)) and a simplified AHSV-3 VP5 antigenicity plot according to parameters of Hopp and Woods (1983). (a(iii)). The grey regions indicate possible interesting areas in the AHSV VP5 protein. The percentage amino acid conservation of these grey areas is indicated in each grey area below the main block.

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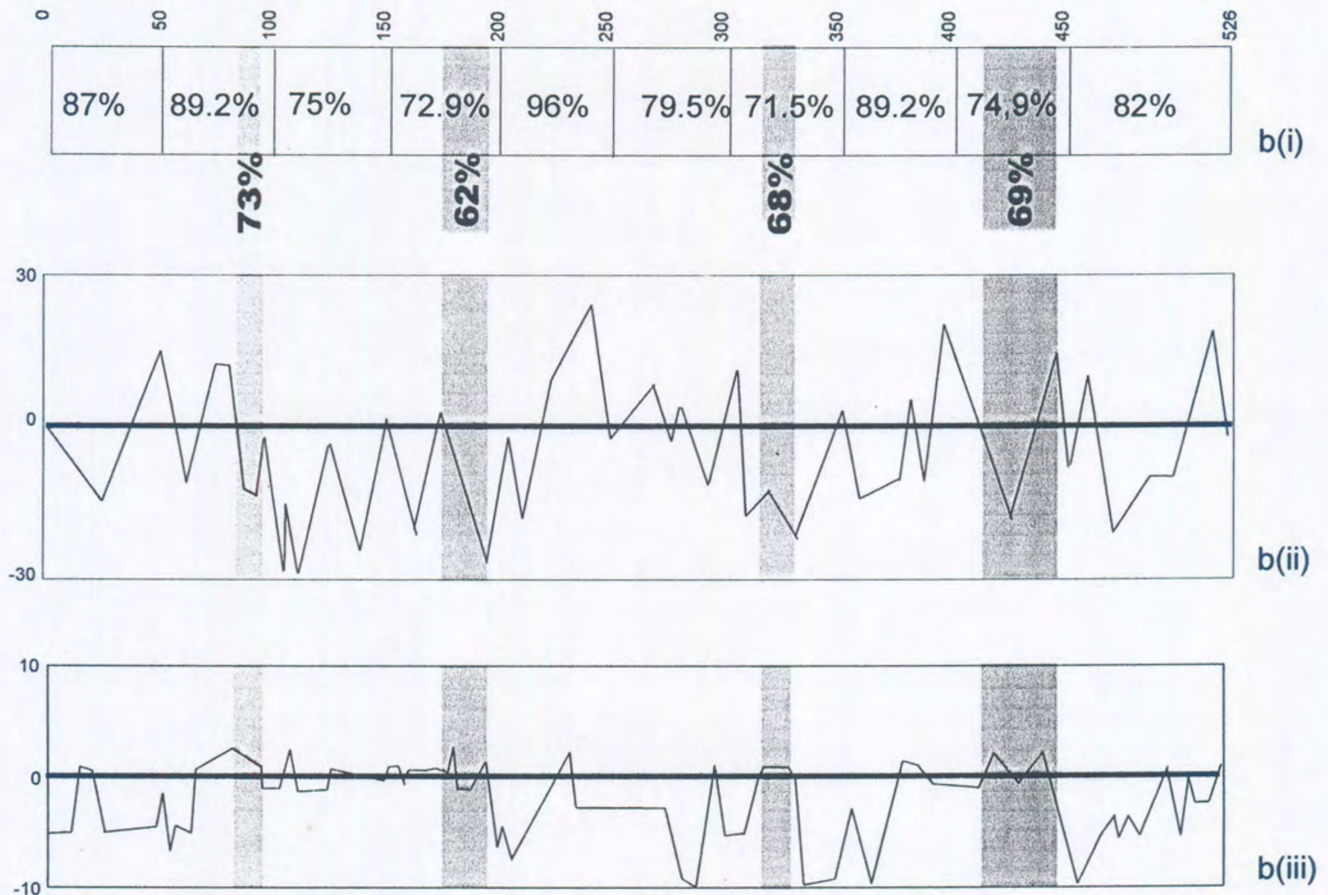

Figure 3.9 (b): Alignment of BTV VP5 amino acid conservation profile determined form the BTV sequences used in Clustal X alignment (Figure 3.3.7) dividing the BTV amino acid sequences into blocks of 50 amino acids and calculating the average conservation of amino acids within the defined regions (b(i)). The simplified hydrophobicity plot of BTV-10 VP5 according to Kyte and Doolittle hydrophobicity values (1982 (b (ii)) and the simplified BTV-10 VP5 antigenicity plot according to parameters of Hopp and Woods (1983) (b(iii)). The grey regions indicate potentially interesting areas in the BTV VP5 protein. The average amino acid conservation for each grey region in indicated in each region below the main block.

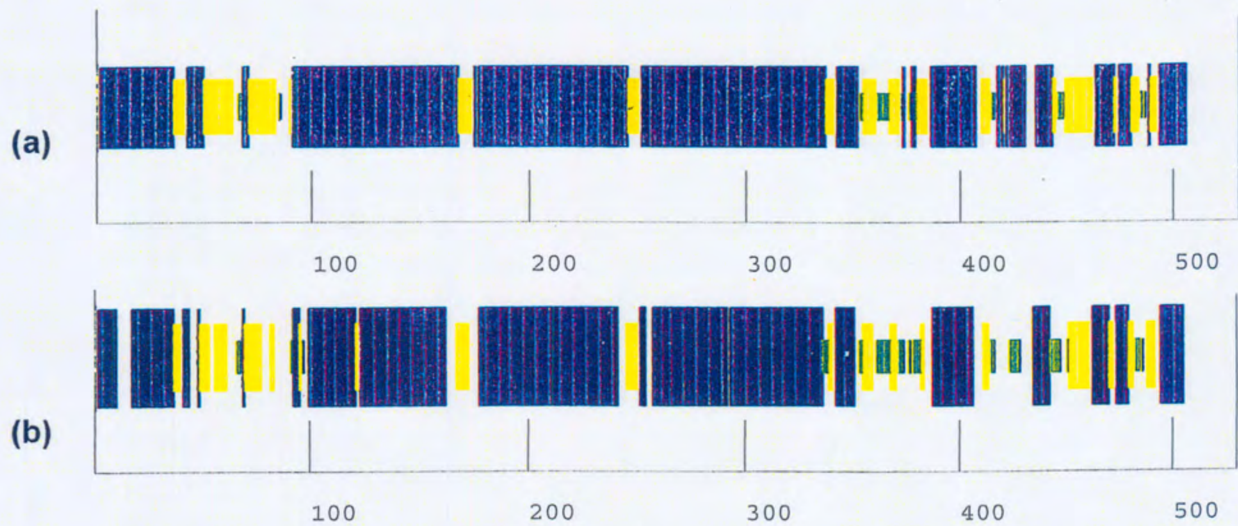

Figure 3.10: Predicted secondary structure of AHSV-3 (a) and AHSV-6 (Williams *et al.*, 1998)(b) VP5 proteins. Dark blue bar graph and regions represent α-helix, yellow graph and regions represent β- sheets and the green graph regions represent turns. The white areas represent regions of unknown secondary structure.

3.4 Discussion

To develop a better understanding of the role of VP5 of AHSV the gene of AHSV-3 VP5 was characterised and compared with known VP5 sequences of orbiviruses. From this nucleotide sequence the amino acid sequence was derived and possible important regions on the VP5 protein were identified.

The nucleotide sequence of the VP5 gene segment of AHSV-3 encoding the VP5 proteins was determined to be 1566bp (Figure 3.3) in length with a base pair composition of 33.2% A, 24.0% T, 16.7% C and 26% G. The length and base pair composition compare well with the known AHSV VP5 nucleotide sequences. The AHSV-4 VP5 gene has a nucleotide sequence of 1566 bp with a compatible base pair composition (32.95% A; 22.3%T; 17.69%C; 26.3%G) (Sakamoto *et al.*, 1994). AHSV-9 VP5 gene sequence also supports these results with a length of 1566bp and a similar base composition (32.8% A; 23.2% T; 17.0%C; 26.9% G) (du Plessis & Nel, 1997).The sequence of AHSV-6 VP5 gene is slightly shorter, 1564bp in length, with compatible base composition (33.1% A; 23.7% T; 16.8% C 26.3% G) (Williams *et al.*, 1998). The base composition of AHSV-6 VP5 is closest to that of the AHSV-3 VP5 gene.

The 5' and 3' terminal sequences of the AHSV-3 VP5 gene, 5' GUUUAU 3' and 5' ACAUAC 3' respectively are completely conserved amongst the known AHSV VP5 nucleotide sequences (du Plessis & Nel, 1997, Iwata *et al.*, 1992a, Sakamoto *et al.*, 1994) with the exception 3' terminal sequences of the AHSV-6 VP5 gene where an additional terminal C in the 3' terminal region (5'ACAUAC**C**3') (Williams *et al.*, 1998) is found. The terminal sequences also are compatible with the AHSV specific 5' and 3' terminal sequences of 5'GUU(A/U)A(A/U)-3' and 5'-(C/A)C(U/A)UAC 3' respectively as described by Mizukoshi (Mizukoshi *et al.*, 1993).

Clustal X alignments were used for the comparison of AHSV-3 VP5 nucleotide sequence to known AHSV VP5 nucleotide sequences (Figure 3.5). Conservation between the nucleotide sequence of AHSV-3 VP5 and AHSV-9 VP5 genes is the highest at 93% followed by a 91% nucleotide sequence conservation between AHSV-3

VP5 and AHSV-6 VP5 genes and a 88% conservation between AHSV-6 VP5 and AHSV-9 VP5 genes (Table 3.1). The conservation between the AHSV-4 VP5 nucleotide sequences and those of AHSV-3 VP5, AHSV-6 VP5 and AHSV-9 VP5 genes were the lowest. These results confirm earlier indications that AHSV VP5 genes are conserved (Bremer *et al.*, 1990).

The average conservation of the nucleotide sequence of AHSV VP5 genes seems very high across the length of the gene. One can however identify a region of higher variability stretching from nucleotides 956-1080 (Figure 3.5). This variable region encodes amino acids ranging from approximately 310-360 (Figure 3.6). Within this region a small section was identified ranging from amino acid 319-325 which is highly variable (Figure 3.6). This highly variable region also coincides with a hydrophilic peak and has been identified as a potentially antigenic (Figure 3.8 (a)). One possible explanation is that this region is expressed on the surface of the virus and that it could contain antigenic sites or epitopes. This could allow greater variation to occur as a result of selective immunological pressure.

Another striking feature of the nucleotide sequence is the high conservation for the first 50 nucleotides (92.8% average) and last 40 nucleotides (95.6% average) of the AHSV VP5 genes. These regions contain the AHSV specific 5' and 3' terminal sequences as described by Mizukoshi (1993) as well as the Kozak regions around the start codon. The end terminal and Kozak regions account only for a small section of the highly conserved ends of the gene. These longer highly conserved regions may play a role in mRNA secondary structure that serve as recognition sites for RNA binding proteins such as NS2, during virus assembly. This hypothesis had been postulated for the highly conserved 3' nucleotide regions of BTV VP7 gene and in segment 1 (Kowalik & Li, 1989) but no specific evidence has yet been found for this hypothesis in the case of BTV or other orbiviruses. For reoviruses it was found that the terminal sequences of the S2 gene were disproportionally highly conserved. This suggested that these sequences are required for maintenance of secondary mRNA structure. A panhandle heteroduplex with strict structural conservation amongst twelve S2 genes was identified with possible functional importance of perhaps selective packaging or

regulation of transcription and translation (Chappel *et al.*, 1994). The 3' and termini consensus sequence of the rotavirus mRNA was shown to be important in replication of the RNA possibly containing a signal for replication. Studies have also shown that at least two types rotavirus signals, possibly based on secondary structure of the mRNA are required for minus-strand synthesis. The signal residing at the 5' terminal seems to lie within the first 87 nucleotides of the mRNA. The rotavirus gene 8 mRNA was predicted to form a panhandle secondary structure with a 3' single stranded tail remaining shown to be necessary for minus-strand synthesis during virus replication (Chen & Patton, 1998, Chen *et al.*, 1994). This may correlate with the highly conserved 50bp nucleotide sequence of AHSV VP5 genes. Further research based on mRNA secondary structure modeling and mutational analysis will be necessary in order to determine if similar systems of control for translation exist for AHSV and other orbiviruses. This should also be investigated for all AHSV genes to determine if a universal pattern could be identified.

Another possible explanation may be that the highly conserved regions may play a role in regulation of translation. For reovirus mRNA it has been suggested that mRNA secondary structure may play a role in translational control (Roner *et al.*, 1989). No direct evidence has however been presented for this hypothesis. In orbiviruses an indication that translational control may exist is suggested by findings of Van Dijk and Huismans (1991). They demonstrated both with *in vitro* translation and radiolabling of proteins in BTV infected cells that the VP5 protein levels were significantly lower than the expected levels with regard to the molar concentration of the transcribed mRNA (Van Dijk & Huismans, 1988). Transcriptional control may be a possible explanation for this observation. The conserved nucleotide regions can therefore play a role in translational control of VP5. This hypothesis is however highly speculative and no direct evidence is available to substantiate this hypothesis. Further investigation may however reveal interesting facts of translational control of AHSV proteins.

The longest open reading frame of the AHSV-3 VP5 nucleotide sequence begins at the ATG in position 20-22 and is terminated in by a TGA in position 1535-1537. The start codon is flanked by a 5' CCAGAG<u>A</u>CC**ATG**<u>G</u> 3' conforming with the

Kozak sequence of which the G^{+4} and the -3 purine are present. These sequences seem to contribute to efficient translation (Kozak, 1989, Kozak, 1991). A second in phase start codon can be identified in position 98-100 but this codon does not comply with the optimal Kozak flanking region due to the absence of the essential G^{+4} (Kozak, 1989). Other internal in phase start codons have also been identified on the AHSV-3 VP5 gene. Three of the start codons at nucleotide positions 107, 461 and 914 contain the G^{+4} and -3 purine of the Kozak sequence. These internal start codons may offer an explanation for the observed second, smaller proteins formed by *in vitro* translation of the AHSV-4 VP5 gene (Martinez-Torrecuadrada *et al.*, 1994) as well as the smaller proteins observed from the *in vivo* expressed AHSV-9 VP5 (du Plessis, 1995). This may be an artifact of the rabbit reticulocyte *in vitro* translation system which has a tendency to 'see' internal initiation sites (Kozak, 1989).

A protein of 505 amino acids is encoded by the longest open reading frame of AHSV-3 VP5 gene. This represents a protein of 56.94 K in size with a pI of 6.30. The size is in agreement with the observed of AHSV-4 VP5 protein (Sakamoto *et al.*, 1994) and AHSV-9 VP5 protein (du Plessis & Nel, 1997) of 505 amino acid peptides. The AHSV-6 VP5 protein consists of 504 amino acids (Williams *et al.*, 1998) one less amino acid than AHSV-3 VP5 protein. The VP5 amino acid composition of the known AHSV VP5 proteins is very similar (Table 3.2). An interesting difference is the variable number of cystein residues. Cysteins form disulfide bonds and play a role in the formation of secondary and tertiary structure of proteins (Stryer, 1988). AHSV-3 VP5 and AHSV-6 VP5 genes contain three cysteins while AHSV-9 and AHSV-4 genes have four and two cystein residues respectively. The conserved cysteins (Figure 3.6 indicated by a *) seem to be found in positions 370 and 483 while another cystein in position 373 seems to be conserved amongst VP5 genes of serotypes 3, 6 and 9. It also corresponds with a conserved cystein in relative position 380 of BTV as seen in Clustal X alignment of known BTV and AHSV sequences in Figure 3.7 indicated by a *. These cysteins found in positions 370 and 483 may play a significant role in the structure of AHSV VP5 proteins.

Clustal X alignment matrix was used for the alignment of amino acid sequences of known AHSV VP5 genes (Figure 3.6, Figure 3.7, Figure 3.8). From the above-mentioned alignments identities and similarities were determined. Identities of 95% between AHSV-3 VP5 and serotype-6 VP5, 90% between serotype-3 and serotype-9 VP5, 83% between serotype-3 and 4 VP5 were determined. Similarities of 98%, 94% and 95% respectively also were calculated (Table 3.3). These results indicate the relatively high conservation amongst AHSV VP5 protein in comparison to the AHSV VP2 protein conservation with an identity of 50.5% and a similarity of 71.3% between serotype-3 and serotype-4 (Vreede & Huismans, 1994). Findings of relatively high conservation were also found amongst BTV VP5 proteins (Iwata *et al.*, 1991, Oldfield *et al.*, 1991). This data illustrates the conserved nature of the orbivirus VP5 protein within a serogroup (Oldfield *et al.*, 1991). To a lesser extend VP5 proteins are conserved amongst the EHDV, BTV and AHSV serogroups (Roy & Sutton, 1998). This was illustrated in Figure 3.8, representing a Clustal X alignment of AHSV and BTV VP5 amino acid sequences.

Amino acid alignments, hydrophobicity and antigenicity plots were used to identify four regions of probable importance (Figure 3.9 (a) and (b)). A comparison of BTV and AHSV VP5 indicated that these regions were located in approximately the same areas on the comparative VP5 proteins. The first region lies approximately between amino acids 80-95 of AHSV-3 and amino acids 85-95 of BTV-10. In both AHSV and BTV these regions have been predicted to elicit B cell antigenic response and correlate with hydrophilic peaks (Figure 3.9). These regions highlighted in grey (Figure 3.9) show amino acid conservation of 84.9% for AHSV and 73.3% for BTV a relatively high level of conservation. This region may therefore be represent possible epitopes but do not seem to be of serotypic importance. On comparison of the BTV and AHSV amino acid sequences in this region the conservation is relatively high. Distinct differences can however be observed between the AHSV and BTV amino acid sequences. If this region contains an antigenic site or epitope it is likely be serogroup specific. The highly conserved prolines amongst both the AHSV and BTV amino acids sequences within this region show that this region may lie in a turn in the protein

structure and therefore the likelihood for an antigenic site or epitope is supported by the probable structure of the protein in this region.

The second region identified is found in the regions of amino acid 175-185 in AHSV and amino acid 170-190 in BTV. These regions are highly hydrophilic, and are also predicted to have the potential for inducing a B cell immune response. The conservation within the known sequence of the AHSV serotypes is very high amongst the known AHSV amino acid sequences (94%) (Figure 3.6; Figure 3.9 (a) i). The BTV VP5 sequences this region is less conserved (61%) (Figure 3.7. and Figure 3.9 b (i)). Yang *et al* identified a linear epitope in this region of BTV VP5. This region was shown to be accessible to monoclonal antibodies when purified, intact BTV was analysed and may therefore be exposed to the surface of the virion (Yang *et al.*, 1992). Therefore this region may be either of structural or antigenic importance for the BTV VP5 proteins. In the case of AHS VP5 proteins the high conservation of this region may indicate a serogroup specific epitope. It may however also indicate high structural conservation for interaction with other virus proteins.

A less conserved area of the AHSV VP5 and BTV coincides with the third interesting region of the VP5 protein. Strong hydrophilic and antigenic peaks suggest a possible antigenic region stretching from amino acids 310-340 with a 76.6% conservation for AHSV and amino acids 310-340 with a 67% conservation for BTV (Figure 3.3.9 (a) and (b)). Evaluation of this region highlights a significantly variable region stretching form amino acid 320-326 AHSV (Figure 3.6) and BTV (Figure 3.7) VP5 amino acid sequences. These variable regions may therefore be of possible serotypic importance in the case VP5. A conserved proline in this region for both BTV (amino acid 326 Figure 3.7) and AHSV (amino acid 325 Figure 3.6), with the exception of AHSV-9 VP5, indicate a possible turn in the area of the VP5 protein. This evidence supports the possibility of an antigenic region. This region may therefore be a serotype specific epitope or play a role in interacting with the unconserved VP2 protein, because it is more variable than the previously identified antigenic regions.

The fourth region of a distinctly lower level of conservation around amino acids 430- 450 with 73.3% conservation for AHSV and 415-445 with 68.6% conservation for BTV. The hydrophilic profile and the predicted potential to elicit B cell immune response correlate within this region. Three 100% conserved prolines can be found in the AHSV VP5 (amino acids in positions 432, 436 and 450) but none have been identified within the BTV region of amino acid 415-445. Prolines often have the function to induce a turn in the secondary protein structure supporting the possibility of a possible epitope for AHSV VP5 (Prescott, 1988). These combined features possibly indicate that this may be an important serotype specific epitopic region on the AHSV VP5 protein.

To determine if the identified antigenic regions are found within a region where a turn is found, the secondary structure of AHSV-3 VP5 was predicated with the aid of the GOR I method (Garnier *et al.*, 1978). From the secondary structure prediction one could deduce that AHSV-3 VP5 consist predominantly out of N-terminal α-helix domain interspersed with a few β-sheets and some coils. The C-terminal region contains β-sheets interspersed with coils dividing the VP5 protein into two distinct regions (Figure 3.10 (a)). This structural prediction is very similar to that of AHSV-9 VP5 , indicating a distinct N-terminal and C-terminal region (du Plessis & Nel, 1997). The secondary structure of AHSV-6 VP5 was also predicted with the use of the GOR I method (Garnier *et al.*, 1978) for comparison with AHSV-3 VP5. The secondary structure profile of AHSV-3 VP5 and AHSV-6 VP5 (Figure 3.10.(b)) were found to be very similar. The predicted secondary structure differs from the secondary structure predicted by Williams *et al* (1998). An N-terminal coil-coil organization and a C-terminal globular structure divided by a hinge region was predicted (Williams *et al.*, 1998). The predicted secondary structure of AHSV-3 VP5 and AHSV-6 VP5 using the GOR I prediction may differ form previously predicted secondary structure of VP5 but in both cases two distinct regions were predicted as described above. The difference in the predicted secondary structure may be a reflection of the different analysis logarithms that were used. From both the GOR I predicted secondary structure and the predicted secondary structure by Williams *et al* (1998) it can be concluded that the VP5 protein most probably consists of two conserved structural domains.

Mertens *et al* (1989) suggested that the VP5 protein may play an important role in serotype determination by proposing that VP5's interaction with VP2 affects the conformational structure and possible serological properties of VP2 (Mertens *et al.*, 1989). The highly conserved regions of VP5 may play a role in maintaining the structural stability of the virion by its interaction with the highly conserved VP7 and the variable VP2 (Williams *et al.*, 1998). The importance of the relatedness of VP2 and VP5 and the virion stability was demonstrated by Loudon *et al* (1991). VLP's consisting of closely related VP2 and VP5 BTV proteins assembled successfully while less related VP2 and VP5 proteins failed to form VLP's (Loudon *et al.*, 1991). VP5 structure therefore seems to play an important role in determining the stability of the outer capsid layer specifically and therefore the virus structure of orbiviruses in general. Through its interaction with VP2, VP5 may therefore also play an indirect role in antigenicity. Another significant observation is that three of the four regions, predicted as possible epitopes according to antigenicity and hydrophobicity plots (Figure 3.8 (a)), fall within a predicted turn of the secondary structure of the AHSV-3 VP5 protein. The first region, although highly conserved, was found around amino acid 90 in the approximate position of a predicted turn (Figure 3.9 (a) and (b)). The second is the region is found from amino acids 320-340 and the third is around amino acid 440. The areas around amino acid 90 and 440 contain three and two 100% conserved proline residues, respectively. This confirms the large possibility of a turn occurring in these areas. The region around amino acid 320 contains one proline which is conserved amongst all peptide except AHSV-9 VP5.

It can therefore be concluded that AHSV VP5 proteins are closely related in amino acid composition and structure. The regions identified above may play a role in either interaction with the variable VP2 protein or the highly conserved VP7 protein. VP5 protein may therefore be a candidate for co-expression with VP2. By co-expressing AHSV-3 VP2 and VP5 proteins, the VP2 protein conformation may be improved to assume a preferred antigenic conformation. Mertens *et al* (1989) have demonstrated that interaction between VP2 and VP5 proteins may be serotype specific. They indicated that VLP's with the two outer capsid proteins from the same or closely

related serotypes are more stable compared to VLP's where VP2 and VP5 from different serotypes. Co- expression of VP2 and VP5 from the same serotype may therefore stabilise VP2 proteins. VP5 may also contribute by presenting serotype or serogroup specific epitopes thereby inducing a more well rounded immune response against AHSV-3 infection.

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CHAPTER 4

Dual Expression of AHSV-3 VP2 and VP5 as baculovirus recombinant proteins

4.1 Introduction

VP5 has been shown to enhance the protective immune response that is elicited by VP2 (Huismans & Van Dijk, 1990, Martinez-Torrecuadrada *et al.*, 1994, Roy *et al.*, 1996, Stone-Marschat *et al.*, 1996). In rotaviruses it was found that VP7 is dependent on other virus proteins to enhance the protective immune response (Dormitzer *et al.*, 1992). In the case of BTV it was shown that VP2 as part of VLP's is approximately ten fold more effective in inducing a protective immune response than VP2 alone (Roy, 1996, Roy *et al.*, 1994a, Roy & Sutton, 1998). These findings all support Mertens' (1989) suggestion that in the presence of VP5 the conformation of VP2 is enhanced to resemble more closely the VP2 found on the virion. Roy (1998) postulated that epitopes of VP2 forming part of a VLP mimic authentic VP2 epitopes found on the virus. VLP's are therefore more effective for vaccination against BTV than VP2 alone. Orbivirus VP2 has the ability to induce protective immune response on its own (Huismans *et al.*, 1987a, Roy *et al.*, 1996). Evidence is however strong that in the presence of other virus proteins the ability of VP2 to induce serotype specific immune response in enhanced. Interaction between VP2 and VP5 proteins seems to be serotype specific (Loudon *et al.*, 1991). It therefore needs to be investigated if co-expression of VP2 and VP5 proteins of the same serotype would enhance the VP2 solubility and ability to induce a neutralising immune response.

For the expression of orbivirus proteins the baculovirus expression system is highly effective (Roy & Sutton, 1998). This system also provided the technology for the dual expression of two proteins. The original system is based on homologous recombination in insect cells to generate recombinant baculoviruses (O'Reilly *et al.*, 1992). Recently the Bac-to-Bac™ baculovirus expression system became available

for expression of proteins in insect cells. This system permits rapid efficient generation of recombinant baculoviruses by site-specific transposition mediated insertion of foreign genes into a baculovirus genome propagated in *E. coli* (Luckow *et al.*, 1993). BACMID, the recombinant baculovirus genome containing the gene of interest is isolated form *E.coli* cells and transfected in to Sf-9 (*Spodoptera frugiperda*) insect cells for the generation of recombinant baculoviruses (Luckow *et al.*, 1993).

Previously, using the single expression vector pFastbac of the Bac-to-Bac™, AHSV-3 VP2 had been expressed in large quantities. It was shown that the baculovirus recombinant AHSV-3 VP2 protein was largely in an aggregated form and therefore possibly not immunogenic. An attempt was made to express AHSV-3 VP2 and VP5 genes simultaneously for the first time with the help of the Bac-to-Bac™ expression system in order to address this problem. Initially AHSV-3 VP5 was expressed using the Bac-to-Bac™ single expression system to ensure successful expression of the protein before attempting the dual expression of the two outer capsid proteins. For the first time the co-expression of the AHSV VP2 and VP5 proteins was attempted with the use of this Bac-to-Bac™ expression system.

4.2 Materials and Methods

4.2.1 In Vitro transcription with T7 RNA polymerase

In vitro transcription was performed using the T7 RNA polymerase (Boehringer Mannheim) as described by the protocol provided. Prepared linearised plasmid (500 ng) was transcribed in a reaction containing 1μl human placental ribonuclease inhibitor (60u/μl Boehringer Mannheim), each dNTP to a final concentration of 1.25mM was added (stock 10mM rNTP, Boehringer Mannheim), 2 μl of 2X buffer provided (0.4M Tris HCl pH 8; 60mM $MgCl_2$; 100mM dithiotreitol (DTT); 20mM Spermidine), 20U RNA polymerase and RNA free water to the final volume of 20μl completed the reaction mix. The reaction was incubated at 37°C for 1h. mRNA transcription was assayed by brief 1% agarose gel electrophoreses. Throughout the procedure care was taken to avoid RNase contamination. Freshly prepared sterile tips and Eppendorf tubes were used. The water was DEPC treated before use (Sambrook *et al.*, 1989). All electrophoresis apparatus were carefully washed with 10% SDS and rinsed with DEPC water before use.

4.2.2 Preparation of double stranded RNA for in vitro translation.

CER cells (American Type Culture Collection ATCC) were obtained form the Onderstepoort Veterinary Institute (OVI). The cells were cultivated in Eagles medium containing 5% Bovine serum (OVI) 1ml of 50 mg/ml Gentamycin, 1ml Penicillin (Novapen 150mg/ml), Streptomycin (150mg/ml) and 1ml Fungizone (Amphotricin B). The cells were divided by tripsinating the cells of the Roux Flasks at 37°C for 5-15 minutes and poured into 1 liter prepared Eagles medium, mixed and divided into 8-10 Roux flasks. The cells were incubated at 37°C for 2-4 days until confluent. The fully grown cells were infected with AHSV virus stock (OVI) at a multiplicity of infection (m.o.i.) grater than 1pfu/cell and incubated at

37° until 80% of the cells were dead (+/- 48hpi). Thereafter the cells were harvested by separating the cells from the flask with sterile glass beads and collecting the cells by centrifugation at 5000g for 30 minutes. The cells were resuspended in 0.15M STE and frozen until dsRNA extraction (Huismans, 1979, Verwoerd, 1969). Double stranded (ds) RNA was extracted using the SDS Phenol method (Huismans & Bremer, 1981) (4.2.3).

4.2.3 Double stranded RNA isolation

Double stranded RNA (dsRNA) was extracted from AHSV infected CER cells (4.2.1) by phenol-chloroform method as described by Huismans and Bremer (1981). CER cells infected with AHSV were resuspended in a final volume of 20ml 0.15M STE. Tris HCl to a final concentration of 2mM, NaAc pH5.0 and EDTA each to a final concentration of 10mM were added. Finally SDS (sodium dodicyl sulfate) was added to a final concentration of 1% w/v in a final volume of 80ml. The pH was adjusted to pH 5.0 with glacial acetic acid before extracting the solution with 0.5 volume phenol at 60°C. The mixture was vigorously shaken before incubation at 60° for 15 minutes, incubated on ice for 15 minutes followed by centrifugation at 10000g. The phenol residues were removed from the aqueous phase with two consecutive chloroform extractions. The dsRNA was precipitated form the aqueous phase in 0.1M NaCl and 2 volumes ethanol. The precipitate was dissolved in DEPC prepared water and 2M $LiCl_2$ was added for overnight precipitation of ssRNA which was pelleted by centrifugation. The supernatant containing the dsRNA was diluted, the dsRNA precipitated with 2 volumes ethanol and thereafter resuspended in DEPC prepared water. The quality of the dsRNA was determined by agarose gel electrophoresis. The dsRNA was enriched for the large fragments by fractionation in a 5-40% a sucrose gradient in TE (10mM Tris pH7.4; 1mM EDTA pH 8.0) centrifuged for 16h at 36 000 rpm in SW 50.1 rotor of the Beckmann Ultra centrifuge. The gradient was fractionated using a gradient tube fractionation (Hoefer Scientific Instruments) and collecting 8-10 drops per fraction. The fractions were analysed by 1% agarose gel electrophoresis. The fractions containing the large dsRNA fragments and fractions containing the small fragments were pooled together respectively. The sucrose was diluted with water to enable the precipitation of dsRNA in 0.3M NaAc pH 8.0 with 2 volumes 96% ethanol.

4.2.4 In vitro transcription of AHSV-3 VP5

To prepare the VP5 recombinant transcription vector the gene was excised from the vector by Bam HI digestion (3.2.3) and ligated (3.2.7) into a Bam HI linearised pBluescript transcription vector (Stratagene). The ligated plasmids were transformed (3.2.9) into competent XL-1 blue cells (3.2.8). Recombinant plasmids containing the AHSV-3 VP5 PCR product were identified by plasmid isolation and verified by restriction enzyme analysis. The orientation of the AHSV-3 VP5 gene was determined by restriction enzyme analysis. The *in vitro* expression vector contains both T7 and T3 RNA polymerase promoters. AHSV-3 VP5 gene was cloned under the direction of the T7 promoter. The recombinant plasmid was linearised with Xba I restriction (3.2.3) at the 3' end of the VP5 gene. The gene was transcribed in vitro using T7 DNA polymerase (4.2.1).

4.2.5 *In vitro* translation of mRNA and dsRNA

mRNA prepared by *in vitro* transcription (4.2.1) and dsRNA of AHSV (total dsRNA; dsRNA enriched for larger fragments; dsRNA enriched for smaller fragments) (4.2.3), denatured with 25mM methyl mercuric hydroxide for 10 minutes at room temperature were translated into proteins using the Rabbit Reticulocite lysate *in vitro* translation system from Amersham Life Sciences. Approximately 500ng each of the prepared mRNA and denatured dsRNA were translated by the following reaction: 2µl of 15.2X translation mix (Methionine free), 2µl 2.5M KAc, 1µl 25mM MgAc and 2µl ^{35}S methionine (Amersham Life Sciences; 10.5mCi/ml) were all added to 10 µl of the Rabbit Reticulocite lysate and mRNA or denatured dsRNA. RNase free water was added to a final reaction volume of 25µl. The reaction was incubated for 90 minutes at 30°C. Translated proteins were analysed by 12% SDS PAGE gel electrophoresis, the gel was dried and the radiolabeled proteins detected by autoradiography (4.2.16).

4.2.6 Cloning into pFastbac transfer vector

For the generation of recombinant baculoviruses, the transfer vectors pFastbac™ and pFastbac Dual™ are available. The pFastbac™ vector contains a polyhedrin promoter under which the gene of interest can be cloned into the appropriate site of the multiple cloning region. The pFastbac Dual™ transfer

vector contains the polyhedrin and p10 promoters. The two late promoters are in a back to back orientation each with its own multiple cloning site for insertion of the gene on interest. All the genetic manipulations were performed in these *E.coli* based vectors before inducing transformation of the gene of interest form the transfer vector into the baculovirus genome, BACMID present in DH-10 Bac cells. The vector is prepared by linearisation with restriction enzyme digests (3.2.3). The gene of interest was also prepared by digestion (3.2.3) and purified (3.2.6) before ligation overnight (3.2.7) into the prepared transfer vector with T4 DNA ligase. The ligation product was transformed (3.2.9) into 100μl competent XL-1 blue cells (3.2.8) using heat shock transformation. After 2 minutes incubation on ice 900μl LB medium was added and the transformed cells were incubated with agitation for 1 h at 37°C. The transformed cells were plated out on agar plates containing 10μg/ml tetracycline, 100μg/ml ampicillin and 3μg/ml gentamycin and incubated overnight at 37°C. Sterile tooth picks were used to pick up a suitable amount of colonies which were grown up in 5ml LB medium containing 10μg/ml tetracycline, 100μg/ml ampicillin and 7μg/ml gentamycin overnight at 37°C with agitation. Plasmids were extracted using the 'miniprep' alkaline lysis method (3.2.1) and recombinants were selected by analysis by 1% agarose gel electrophoresis using a non-recombinant plasmid as control. Recombinant plasmids selected were confirmed and orientation determined by restriction enzyme digest (3.2.3) and 1% agarose gel electrophoresis analysis.

4.2.7 Competent DH10 Bac cells by DMSO method

DH-10 Bac cells containing the BACMID genome and the helper plasmid were grown overnight at 37°C with agitation in 3ml LB medium containing 50μg/ml Kanamycin sulfate and 10μg/ml Tetracycline hydrochloride. One milliliter of the overnight culture was inoculated into 100ml of LB medium containing the relevant antibiotics. The cells were grown to the early logarithmic (log) phase (OD_{550} =0.5) and harvested in a sterile tube by centrifugation at 5000g for 3 minutes at 4°C. The cells were resuspended in $^{1}/_{10}$ of the initial volume of ice cold TSB (LB medium enriched with 10% w/v PEG (Polyethelene glycol, MW 3350); 10mM $MgCl_2$; 10mM $MgSO_4$) and incubated on ice for 10-20 minutes before transformation. The surplus cells were stored in 15% glycerol at -70°C until further use.

4.2.8 Transformation of competent DH10 Bac cells

The recombinant transfer vector pFastbac was transformed into competent DH-10 Bac cells (4.2.6) by adding +/- 500ng of recombinant pFastbac vector to 100μl of competent DH-10 Bac cells and incubating the mixture on ice for 10-30 minutes to allow DNA attachment to the cells. Thereafter 900μl of TSBG (TSB containing 20mM Glucose) was added to the cells and the cells were incubated with agitation at 37°C for 4h. Aliquots of 100μl of the cells were plated out on agar plates containing 50μg/ml kanamycin, 10μg/ml tetracycline; 7μg/ml gentamycin; 40μg/ml IPTG (isopropyl-β-D-thiogalactopyranoside) 300μg/ml X-gal (5-Bromo-4-chloro-3-indonyl-β-D-galactopyranoside). The plates were incubated at 37°C for at least 24h before replica plating white colonies to ensure that the colonies are true white. White colonies represent recombinant BACMID genome containing the gene of interest. The gene of interest first cloned into the pFastbac vector was inserted into the BACMID genome present in the competent DH-10 Bac cells by transposition. Both the pFastbac transfer vector and the BACMID genome contain Tn7 transposition sites. With the help of the genetic information carried by the helper plasmid also present in the competent DH-10 Bac cells, the gene of interest including the gentamycin gene of the pFastbac transfer vector are inserted into the BACMID genome. Successful transposition is indicated by the white phenotype of the DH-10 Bac cells in the presence of X-Gal and IPTG indicating that the Lac Z gene has been interrupted in the BACMID genome and therefore the transposition was successful. The cells containing the recombinant BACMID genome with the gene of interest can then be grown up in cell culture and the BACMID genome extracted for transfection into SF-9 insect cells.

4.2.9 BACMID isolation

True white recombinant DH10 Bac cells were inoculated into 3ml of medium containing 50μg/ml kanamycin, 10μg/ml tetracycline and 7μg/ml gentamycin and incubated overnight at 37° C with agitation. The BACMID genome was isolated by a modified alkaline lysis method (Life Technologies), developed for the extraction of large plasmids (> 100K bp). Cells were collected by centrifugation at top speed for 1 minute in a bench centrifuge. The Supernatant was discarded and the cells resuspended into 300μl buffer containing 15mM Tris HCl pH 8.0 and 10mM EDTA. The cells were lysed by the addition of

300μl of 0.2N NaOH and 1% SDS. The mixture was incubated at room temperature for 5 minutes before 300μl of KAc pH 5.5 was slowly added to precipitate denatured chromosomal DNA and proteins and to renature plasmid DNA. BACMID DNA was separated form the precipitate by centrifugation in a bench centrifuge for 15 minutes at maximum speed. The BACMID containing supernatant was transferred to 0.7 volumes of isopropanol for the precipitation of the BACMID genome. The precipitated plasmid was washed in 70 % Ethanol and resuspended in sterile water. Freezing of the isolated BACMID genome was avoided to prevent mechanical DNA shearing of the BACMID genome.

4.2.10 Cloning of AHSV-3 VP5 into pFastbac™

A PCR copy of the AHSV-3 VP5 gene was excised from the pBluescript vector with BamHI (3.2.3) and purified by agarose gel electrophoresis and Geneclean™ extraction (3.2.6). The gene was ligated into pFastbac™ vector linearised with BamHI (3.2.7). The plasmids were transformed (3.2.9) into competent XL-1 blue cells (3.2.8) and plated out on agar plates containing 10μg/ml tetracycline, 100μg/ml ampicillin and 3μg/ml gentamycin and incubated overnight at 37°C. Plasmids were isolated from randomly selected colonies and recombinant plasmid were identified according to size by agarose gel electrophoresis. Recombinant plasmids were digested with Bam HI to confirm the presence of the gene and with Hind III to determine the orientation of the gene.

4.2.11 Cloning of AHSV-3 VP2 and VP5 into the pFastbac Dual™ vector

The VP2 gene was cloned into the StuI/ Hind III sites under the polyhedrin promoter. The VP2 gene was excised form the pBS vector by digestion with SmaI, Hind III and ScaI (3.2.3), analysed by agarose electrophoresis, followed by purification of the 3200bp VP2 specific gene segment from the agarose gel by Glassmilk™(3.2.6). The gene was ligated directionally (3.2.7.) into the Stu I and Hind III sites of the polycloning region of the polyhedrin promoter pFastbac DUAL™ vector. The VP5 gene was cloned into the SmaI site in under the p10 promoter. For this purpose the gene was excised from the pBluescript vector with Bam HI restriction (3.2.3) followed by blunt ending of the overhangs with Klenow enzyme (3.2.5). The restricted plasmid was analysed by agarose gel electrophoresis and the 1566bp VP5 specific band was purified from the agarose gel by Glassmilk™ purification. The gene was ligated (3.2.7.) into the a SmaI site of the linearised (3.2.3.) and dephosphorilised (3.2.4) pFastbac DUAL™ vector. The two ligation mixes were transformed into competent XL-1 blue cells. Recombinant plasmids were identified by size discrimination from plasmids isolated from randomly selected colonies. The presence of the gene and its orientation were confirmed by restriction enzyme digestion. For co-expression of the two outer capsid proteins a transfer vector containing both VP2 and VP5 genes was constructed. The VP5 gene cloned under the control of the p10 into the pFastbac DUAL vector already containing the VP2 gene. The VP5 gene was excised from the pBluescript vector with BamHI restriction and blunt ended with Klenow enzyme. The restricted plasmid was analysed by agarose gel electrophoresis. Thereafter the 1566bp VP5 specific gene was purified from the agarose gel by Glassmilk™ purification and ligated into the a SmaI linearized, dephosphorilised transfer vector. The ligation plasmid mix was transformed into competent XL-1 Blue cells. Recombinant plasmids containing both genes were identified by size discrimination of plasmid isolated from randomly selected colonies.

4.2.12 Transfection of the BACMID genome into Sf-9 cells

Sf-9 cells propagated in 80cm^3 flasks in modified Grace's medium containing antibiotics and 10% v/v FCS were harvested and seeded at 1X10^6 cells/ well in 35mm 6 well plates (2.2.1). The cells were allowed to adhere to the flasks for at least one hour. The BACMID DNA was prepared for transfection by mixing 5μl of isolated BACMID DNA with 100μof Grace's medium without antibiotics and FCS. Cell Fectin™ (6μl) (Gibco BRL; Life Technologies) was mixed into 100μl of Grace's medium without antibiotics and FCS. The two prepared solutions were combined, gently mixed and incubated at room temperature for 15-45 minutes. The adhered cells were washed twice with Grace's medium without antibiotics and FCS. 800μl of Grace's medium was added and the washed cells were overlaid with the transfection mix containing the diluted DNA-lipid complexes. After 5 hours of incubation at 28°C the transfection mix was removed from the cells and replaced with 2 ml of Grace's medium countering antibiotics and FCS. The cells were incubated for 72 hours at 28°C before the medium containing the recombinant baculoviruses was harvested as first generation virus stock.

4.2.13 Generation of Virus stock

Virus stock of recombinant baculoviruses was grown up by seeding $1x10^7$ cells into a $80cm^3$ flask, allowing the viruses to adhere to the flask for 1h and infecting the cells with first generation virus stock at a low m.o.i.. The cells were incubated for 96h at 28°C before harvesting the medium containing the virus stock. Cells were removed by centrifugation at 3000 rpm for 5 minutes in sterile tubes before filter sterilizing the virus stock and storing it at 4°C until further use. (O'Reilly *et al.*, 1992)

4.2.14 Radioactive labeling of probes by nick translation

Radioactive labeling of DNA was carried out by nick translation using a commercially available kit (Pomega). DNAse 1 introduces nicks into the DNA, these are translated by the exonuclease and polymerase functions of the DNA polymerase 1 enzyme, incorporating radioactive labeled nucleotides. The prepared DNA (500ng) was incubated in a nick translation buffer (50mM Tris pH 7.2; 10mM $MgSO_4$; 0.1mM DTT) with 20μM of each of dCTP; dGTP; dTTP and 10μCi of α^{32}P dATP (>400Ci/mM Amersham), 1 unit of DNA polymerase and 0.2 ng DNAse1 for 1h at 15°C. The reaction was stopped by placing it on ice. The unincorporated nucleotides were separated form the labeled DNA by size exclusion chromatography in a small Sephadex G75 column equilibrated with 1X TE, containing 0.5M SDS. The fractions containing the radioactively labeled DNA were pooled and stored at -20°C until further use.

4.2.15 mRNA blot

The mRNA of the Sf-9 cells were detected using a mRNA blot (Paeratukul *et al.*, 1988). Sf-9 cells were infected with virus stock. The cells were harvested at the appropriate time (24hpi/36hpi),. The cells were washed with 1X PBS and $1X10^5$ cells were diluted into 100μl 1X PBS before dot blotting the cells onto a Hybond-C extra nitrocellulose membrane(Amersham Life Science), pre-wetted with high quality water, using the 'BIO-DOT' dot blotter (BIO RAD). The cells were fixed to the membrane using 3% NaCl; 10mM NaH_2PO_4; 40mM $Na_2H_3PO_4$ pH 7.4; 15% glutaraldehyde solution for 1 hour at 4°C. The fixed cells were rinsed thrice in proteolytic buffer (50mM EDTA, 0.1M Tris HCl pH 8.0) and the protein digested by incubation in proteolytic buffer containing 20μg/ml of Proteinase K (Boehinger Mannheim) at 37° for at least 30 minutes. The membrane was air dried briefly before hybridization.

4.2.16 Blotting of dsDNA onto membrane

Denatured dsDNA was blotted onto a Hybond-C extra nitrocellulose membrane(Amersham Life Science), pre-wetted with high quality water, using the 'BIO-DOT' dot blotter (BIO RAD). The spots where the dsDNA was spotted was prepared by first blotting 20X SSC (3M NaCl; 0.3M Na_4 Citrate.$2H_2O$ pH 7.4) onto the membrane. The double stranded DNA was denatured in O.4 NaOH, 0.4 M EDTA and spotted onto the membrane followed by 100μl of 20x SSC. The DNA was fixed to the membrane by exposure to UV light for 5 minutes on each side of the membrane on the UV transilluminator before hybridization.

4.2.17 Hybridisation

The membrane with fixed nucleic acids was prehybridised in hybridisation buffer (0.75M NaCl; 0,5M $NaH_2PO_4 \cdot H_2O$; 5mM EDTA pH7.4; 50% deionised formamide; 0.2% w/v SDS; 1% w/v Fat Free Milk powder) at 42°C for at least 30 minutes. Probes prepared by nick translation (4.2.11) were denatured with $^1/_{10}$ volume 1M NaOH and heat (94°C for 5 minutes) and transferred onto ice followed by the addition of $^1/_{10}$ volume 1M HCl. The denatured probe was added to the hybridisation mixture and the membrane was incubated at 42°C for 16h. The probe was removed and stored at -20°C for further use. The membrane was washed twice for 10 minutes in 2x SSC (0.3M NaCl; 30mM Trisodium citrate; pH 7.4) followed by a 15 minute wash at room temperature in 2X SSC containing 0.15% SDS. If it was necessary to increase stringency, the membrane was finally washed twice in 2X SSC at 65°C followed by a wash with 1x SSC at 65°C. The access liquid was blotted from the membrane and the membrane was sealed in a plastic bag before detection of the hybridisation by autoradiography using a X-Ray film (Cronex^R MRF medical X-Ray Film) (4.2.16).

4.2.18 Radiolabeling and SDS PAGE gel analysis of viral proteins

To determine if protein was expressed as a baculovirus recombinants in insect cells, the proteins were radioactively labeled *in vitro* using ^{35}S Methionine (Amersham Life Sciences, 10.5mCi/ml). Sf-9 cells seeded at $1X10^6$ cells/35mm well were infected with recombinant baculovirus at a high titer to ensure synchronized infection of the cells. The cells were incubated at 28°C for 24-36 h. The medium was removed from the cells and the cells washed with methionine free Eagles medium (Highveld Biological PTY Ltd.) before incubation the cells in the medium for 1h to starve the cells form methionine. One microliter of ^{35}S labeled methionine was added to the cells and the cells were incubated for a further four hours at 28°C. The cells were harvested and washed twice in 1X PBS before resuspending the cells in 1X PBS. An aliquot of the radiolabeled cells was added to an equal volume of 2X PSB (Protein solvent buffer 0.125M Tris HCl pH 6.8; 4% w/v SDS; 20% v/v glycerol; 10% v/v β mercapto ethanol) and heated to 95°C for 5 minutes before sonification for 10 minutes. The samples were analysed by 12 or 15% SDS PAGE gel electrophoresis as described by Sambrook et al (1989)(2.2.3). The gel was dried and autoradiographed (4.2.16) for visualisation of the proteins.

4.2.19 Autoradiography

Polyacrylamide gels with radioactive samples were dried in a slab gel drier (Hoefer Scientific) for 1h at 60°C under vacuum. The dried gel was exposed to Cronex MRF X-ray film for the appropriate length of time. In case of ^{32}P labeled samples, an intensifying screen was used and the exposure was conducted at -70°C

4.3 Results

All methods used to obtain the following results were described in section 4.2. unless otherwise stated.

4.3.1 *In vitro* translation of AHSV-3 VP5

The first step in the expression of AHSV VP5 was to verify that the PCR clone of the VP5 gene provided by Frank Vreede from the Onderstepoort Veterinary Institute (OVI) encoded a protein of expected size of 56K. To provide the necessary proof, the gene was first cloned into the pBluescript transcription vector followed by *in vitro* transcription into mRNA and mRNA was translated into protein. Denatured double stranded AHSV RNA preparations, isolated by phenol-chloroform extraction and enriched for either the large or small dsRNA segments, were used as *in vitro* translation control. The translation products of AHSV-3 VP5 specific mRNA and the denatured dsRNA were radiolabeled with ^{35}S Methionine, analysed by 12% SDS PAGE gel electrophoresis and visualised by autoradiography.

The results are shown figure 4.1. Lane 1 represents the transcribed total dsRNA of AHSV-6. With the exception of the largest protein VP1, most of the proteins of the AHSV can be seen lane 1. Lane 2 represents the dsRNA enriched for the large proteins mainly consisting of proteins VP1 to VP5 and a small number of the smaller proteins. Lane 3 represents *in vitro* translated smaller dsRNA segments. The *in vitro* translated VP5 protein can be see in all three lanes. A negative control in lane 4 shows the expected background. The translation products of AHSV VP5 specific mRNA are seen in lane 5. The largest band corresponds very closely to the AHSV VP5 proteins of the positive controls. The slight retardation of the VP5 in lane 5 could be due to protein aggregates in the well. A large number of smaller background bands can also be seen in lane 5. The most likely explanation for the smaller bands are the numerous internal start codons found on the AHSV-3 VP5 gene. These are represented in Figure 4.2 and will be discussed in more detail in the discussion. However a protein of the expected size was translated *in vitro* from the mRNA. This indicated that the longest open reading frame encoded for a product corresponding to the expected size of VP5.

4.3.2 Cloning of AHSV-3 VP5

For the expression of AHSV-3 VP5 gene in Sf-9 insect cells a recombinant baculovirus carrying the VP5 gene was generated. A copy of AHSV-9 VP5 was similarly cloned into the pFastbac™ vector as a control. The recombinant pFastbac transfer vectors were used to generate recombinant BACMID shuttle vectors. The recombinant pFastbac plasmids were transformed into competent DH-10 Bac cells containing BACMID DNA and a helper plasmid. The VP5 gene together with the gentamycin resistant gene was transferred to BACMID DNA by transposition. White colonies containing recombinant BACMID were selected. The recombinant BACMID DNA was isolated from *E. coli* cells and transfected into Sf-9 cells. Virus stocks of AHSV-3 VP5 recombinant baculovirus and AHSV-9 VP5 recombinant baculovirus were harvested and further evaluated in order to verify protein expression.

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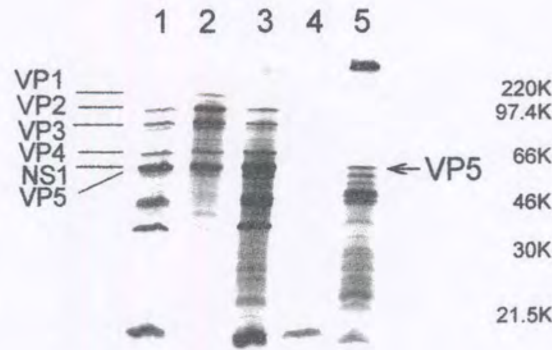
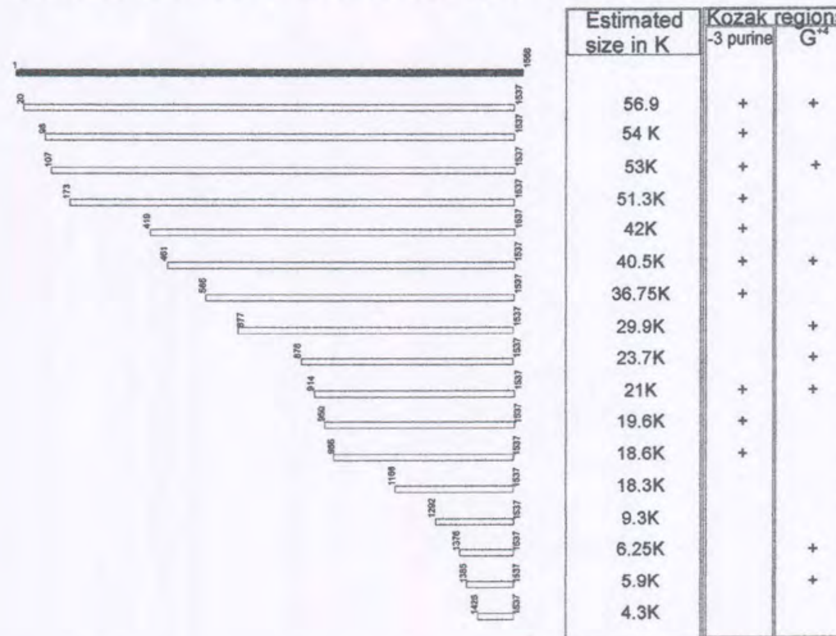


Figure 4.1: Autoradiograph of *in vitro* translation of AHSV-3 VP5 mRNA and AHSV dsRNA analysed on a 12% SDS PAGE gel .

Lane 1: *In vitro* translation of total dsRNA of AHSV-6.
Lane 2: *In vitro* translation of AHSV-6 ds RNA enriched for larger segments.
Lane 3: *In vitro* translation of AHSV-6 drRNA enriched for smaller segments.
Lane 4: Negative control.
Lane 5: *In vitro* translation of AHSV-3 VP5. VP5 indicated by arrow.



Estimated size in K	Kozak regions	
	-3 purine	G^{+4}
56.9	+	+
54 K	+	
53K	+	+
51.3K	+	
42K	+	
40.5K	+	+
36.75K	+	
29.9K		+
23.7K		+
21K	+	+
19.6K	+	
18.6K	+	
18.3K		
9.3K		
6.25K		+
5.9K		+
4.3K		

Figure 4.2: A graphic representation of the AHSV-3 VP5 gene and the ORF's identified in the gene. The black bar represents the complete AHSV-3 VP5 sequence. The bars below represent the different ORF's. The longest ORF encoded the AHSV-3 VP5 gene. The other ORF's all encode 'truncated' AHSV-3 VP5. The position of each start and stop codon is given as well as the predicted molecular weight of the peptide is also given. The presence of the nucleotides of the Kozak region are indicated by + for each start codon. Apart from the longest ORF three other start codons are also flanked by the Kozak sequence

4.3.3 *In vivo* transcription of AHSV-3 VP5 mRNA

The *in vivo* transcription of AHSV-3 VP5 specific mRNA was verified by dot blotting and detection with a radiolabeled AHSV-3 VP5 specific probe as indicated in Figure 4.3. The specificity of the VP5 probe was verified by the successful hybridisation of the probe to the positive control (1) and that the probe did not hybridise to the negative controls (2), (3) and (8). The successful cloning of the gene was confirmed by the hybridisation of the probe to (6) and (7). The probe hybridised to the mRNA from Sf-9 insect cells infected with recombinant baculovirus expressing AHSV-3 VP5 (4) and AHSV-9 VP5 (5). This indicated the successful transcription of the VP5 specific mRNA in the baculovirus infected insect cells. Protein expression of AHSV VP5 was therefore expected.

4.3.4 AHSV-3 VP5 protein expression in Sf-9 cells

The expression of VP5 of AHSV-3 in insect cells infected with recombinant baculovirus carrying the AHSV-3 VP5 gene was verified following the successful detection of serotype 3 VP5 specific mRNA. Insect cells infected with recombinant baculovirus carrying the AHSV-3 VP5 gene were harvested 72hpi and analysed by 15% SDS PAGE. The Coomassie blue stained gel (Figure 4.4) indicates the AHSV-3 VP5 protein is present as a faint unique band (lane 5) obscured by a broad cellular band. No corresponding bands can be seen in either the mock infected cells (lane 2), the wild type infected cells (lane 3) nor the cells infected baculovirus carrying the AHSV-3 VP2, showing VP2 expression at 111K (lane 4). Unfortunately the cellular band above the VP5 band at 56K made it difficult to see the VP5 clearly. To confirm this result the AHSV-3 VP5 protein was radiolabeled at 30-34hpi in order to attempt better visualisation of the VP5 band. The proteins were analysed by SDS PAGE and autoradiography (Figure 4.5). A unique band can be seen in the expected position of AHSV-3 VP5 of 56K (Lane 4). This corresponds to the VP5 protein size produced by *in vitro* translation double stranded AHSV RNA (Lane 5). AHSV-3 VP5 protein has therefore been successfully expressed in insect cells infected with recombinant baculovirus.

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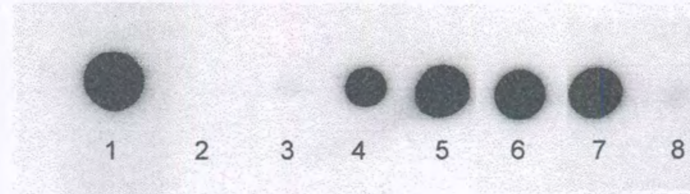

Figure 4.3: DNA and mRNA dot blots hybridised with a VP5 specific probe to detect either the presence of the VP5 gene in the indicated vector or VP5 specific mRNA produced in Sf-9 cells infected with baculovirus recombinants.

1. AHSV-3 VP2 DNA in pBS.
2. Mock infected cells.
3. AHSV-3 VP2 DNA in pBS.
4. Insect cells infected with AHSV-3 VP5 recombinant baculovirus.
5. Insect cells infected with AHSV-9 VP5 recombinant baculovirus.
6. pFastbac AHSV-3 VP5 DNA
7. AHSV-3 VP5 BACMID DNA
8. BACMID DNA

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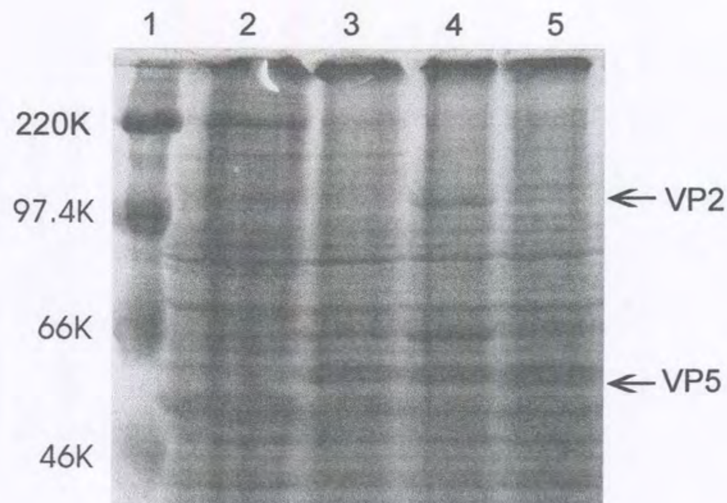

Figure 4.4: Analysis of protein expressed in Sf-9 insect cells infected with different baculoviruses (as indicated in lanes 1-5) by 15% SDS PAGE stained with Coomassie blue.
Lane 1: Protein size marker.
Lane 2: Mock infected cells.
Lane 3: Wild type baculovirus infected cells.
Lane 4: AHSV-3 VP2 recombinant baculovirus infected cells.
Lane 5: AHSV-3 VP5 recombinant baculovirus infected cells.

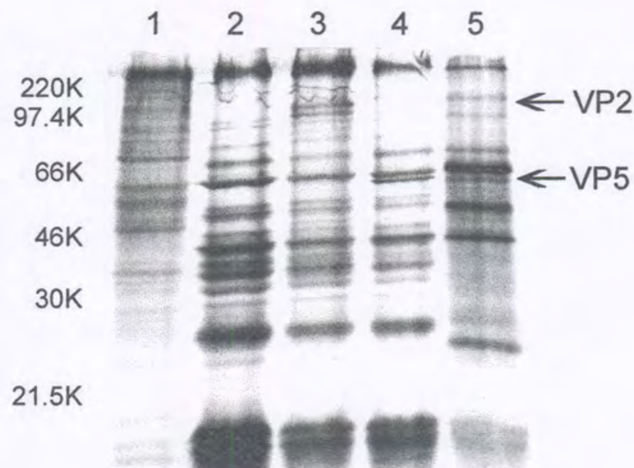

Figure 4.5: Autoradiograph of 15% SDS PAGE gel anlaysis of radiolabeld proteins in Sf-9cells infected with different baculoviruses (as indicated in lanes 1-5).
Lane 1: Mock infected
Lane 2: Wild type baculovirus infected Sf-9
Lane 3: AHSV-3 VP2 recombinant baculovirus
Lane 4. AHSV-3 VP5 recombinant baculovirus
Lane 5. *In vitro* translated dsRNA

4.3.5 Cloning and dual expression of AHSV-3 VP2 and AHSV-3 VP5

The AHSV-3 VP2 and VP5 genes were cloned into the pFastbac DUAL™ transfer vector to co-express the two proteins in insect cells. The VP2 and VP5 genes were first cloned separately into pFastbac DUAL™ vector to ensure that both the polyhedrin and p10 promoters were functional. The VP2 gene was cloned under the polyhedrin promoter (Figure 4.6 (a)) and the VP5 gene was cloned under the p10 promoter (Figure 4.6 (b)). The VP5 gene was thereafter cloned under the p10 promoter of the recombinant pFastbac DUAL vector containing the VP2 gene cloned under the polyhedrin promoter for co-expression of the two genes (Figure 4.6 (c)).

The successful cloning of the two genes is illustrated in Figure 4.7. The VP2 pFastbac DUAL recombinant was confirmed by linearisation with Hind III yielding a 8537bp linearised plasmid (Figure 4.7. lane 3). The orientation of the gene was confirmed by KpnI restriction. The 800bp and 7437bp bands (Figure 4.7. lane 4) indicate the correct orientation of the VP2 gene in pFastbac DUAL. The VP5 gene presence and orientation was confirmed by restriction with Bgl II yielding three expected fragments of 470bp, 2179bp and 4167bp (Figure 4.7 lanes 5 and 6). Recombinant plasmids were confirmed by restriction with Hind III yielding bands of 4800bp and 5500bp (Figure 4.3.7 Lane 7). These results indicate that both the VP2 and VP5 genes were successfully cloned in the correct orientation into the pFastbac DUAL™ transfer vector.

To minimise deletions experienced during the cloning of the two genes into the transfer vector, the recombinant plasmid was placed under selective pressure as soon as possible. During a brief incubation period, immediately after transformation when plasmid replication takes place, the cells were plated out on agar containing full concentration of ampicillin (100μg/ml) and tetracycline (10μg/ml) and half of the recommended gentamycin concentration (3.5μg/ml). The addition of gentamycin to the LB agar medium was a slight modification of the original protocol which did not include gentamycin in the LB agar at this stage. Only half of the general recommended

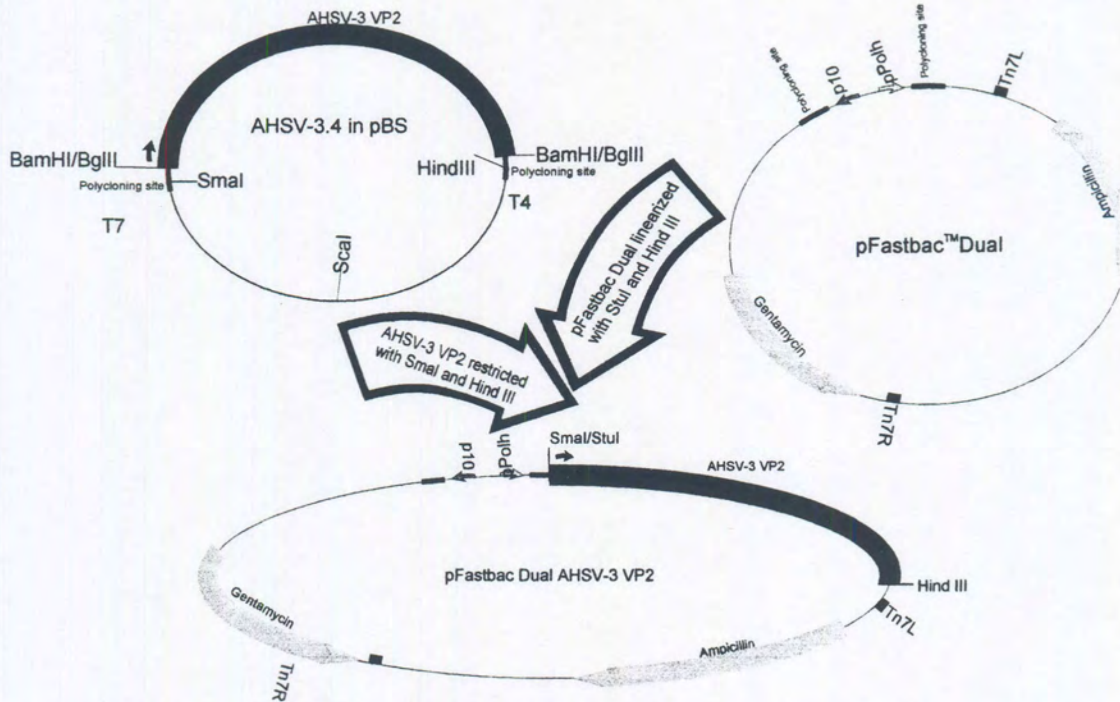

Figure 4.6(a): Cloning strategy for AHSV-3 VP2 gene into pFastbac Dual under the polyhedrin promoter.

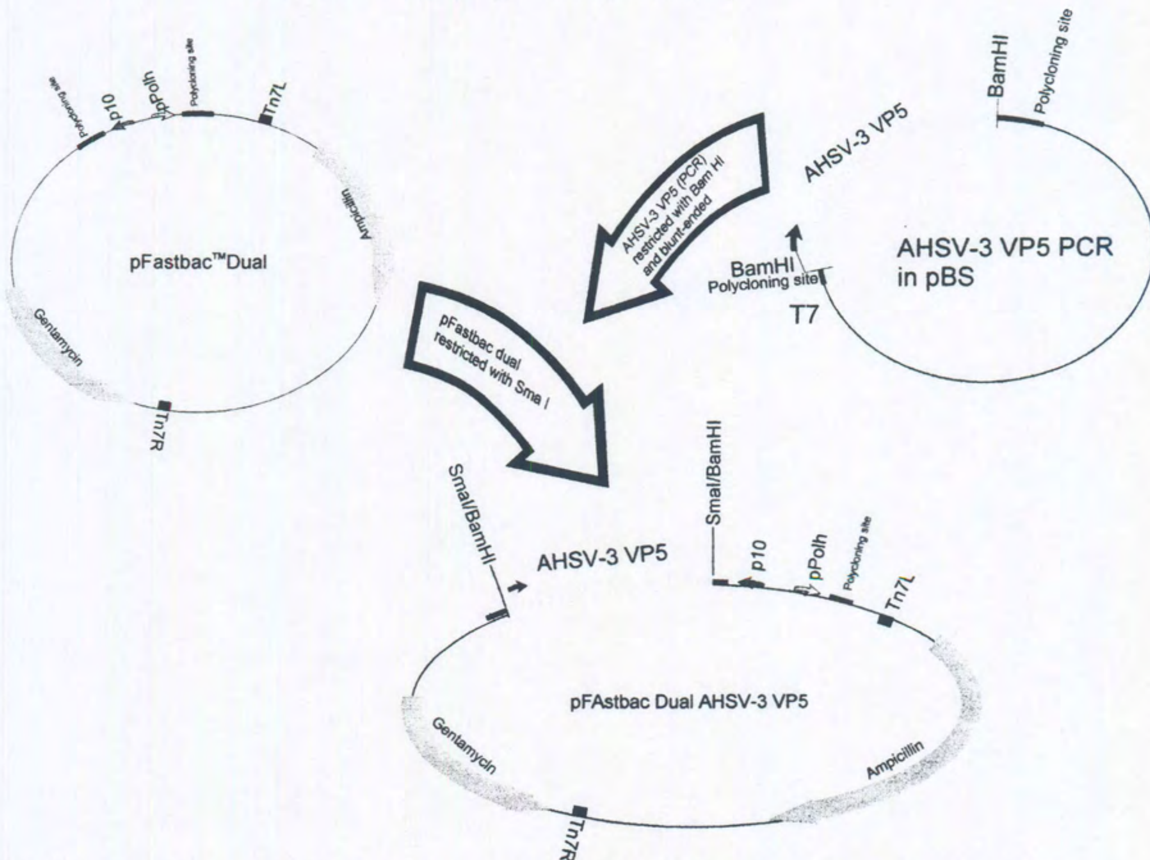

Figure 4.6 (b): Cloning strategy of AHSV-3 VP5 gene into pFastbac Dual under the p10 promoter.

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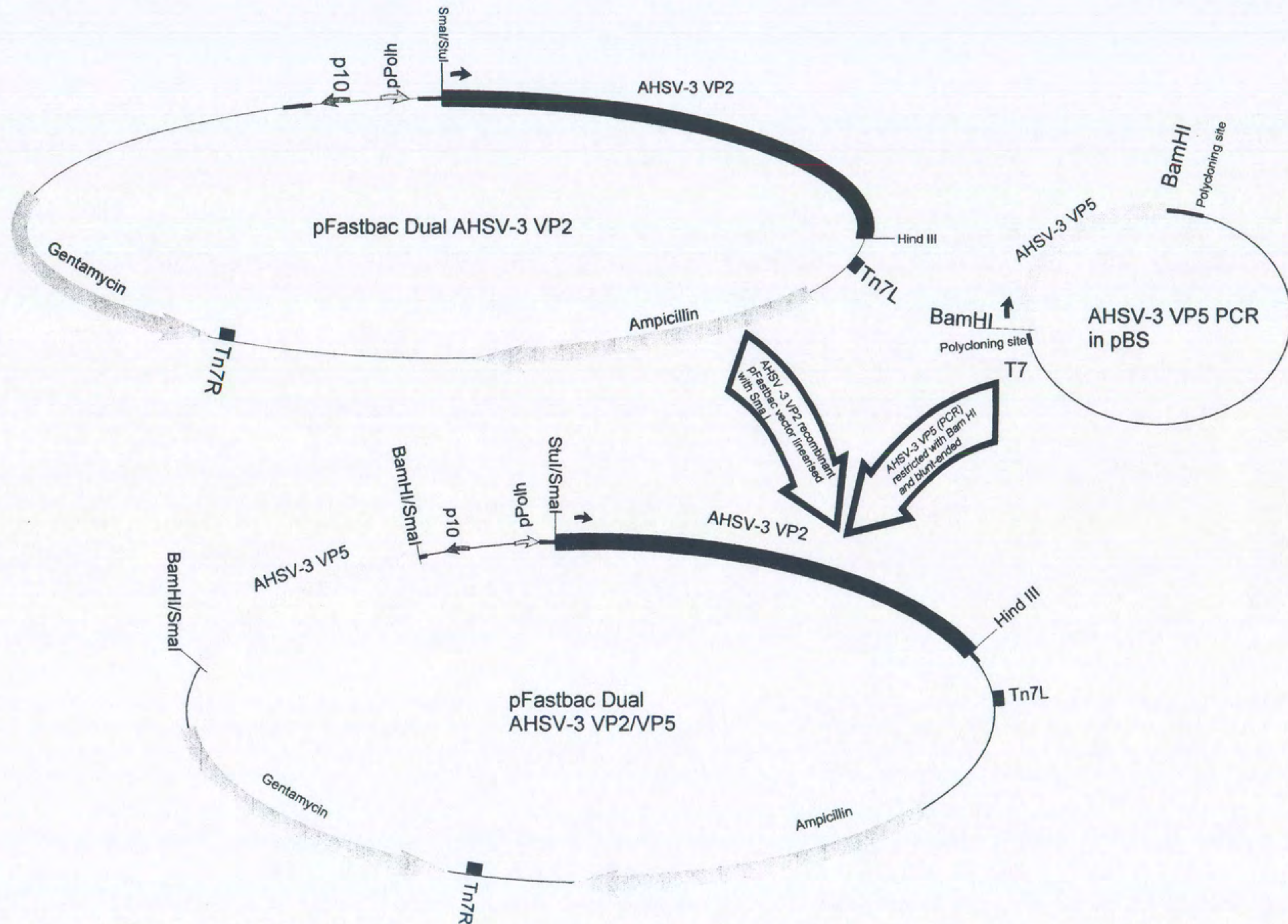

Figure 4.6(c): Cloning strategy of AHSV-3 VP5 gene into recombinant AHSV-3 VP2 pFastbac dual plasmid under the control of the p10 promoter.

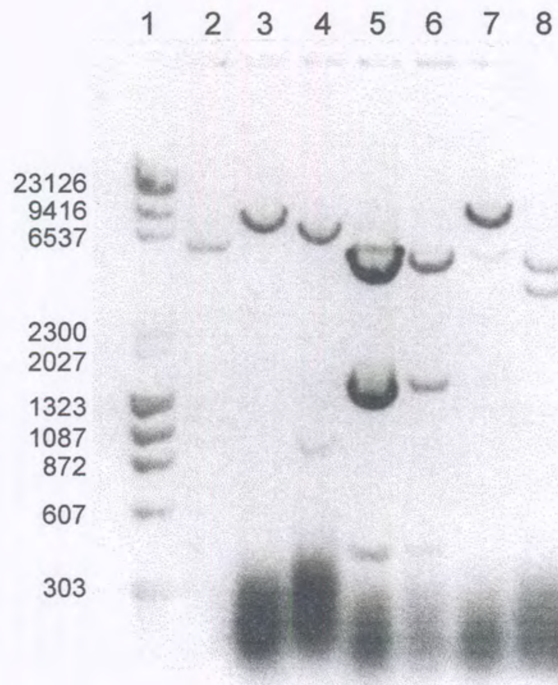

Figure 4.7: 1% agarose gel of restriction enzyme analysis of different pFastbac dual recombinants containing VP2, VP5 and VP2 and VP5 genes of AHSV-3 cloned into the pFastbac Dual™ transfer vector.

Lane1: Molecular weight markers θX and MW II (sizes indicated in base pairs)
Lane 2: Linearised pFastbac Dual
Lane 3: VP2 in pFastbac Dual linearised with Hind III
Lane 4: VP2 in pFastbac Dual restricted with KpnI
Lane 5: VP5 in pFastbac Dual (clone 1) restricted with BglII
Lane 6. VP5 in pFastbac Dual (clone 2) restricted with BglII
Lane 7: VP2 and VP5 in pFastbac Dual restricted with Hind III
Lane 8: VP2 and VP5 in pFastbad Dual restricted with KpnI

gentamycin concentration was used because at a gentamycin concentration of 7μg/ml the no colonies were observed to be due to the late expression of the gentamycin resistance gene in the cells. The selected cells were grown up in 5 ml cultures with the full concentration of all antibiotics. A large number of colonies were screened to ensure the isolation of a recombinant that did not contain deletions. After the selection of a recombinant plasmid, the selected plasmid was immediately used for transformation into competent DH10 Bac cells. It was found that if the cells containing recombinant plasmid were propagated further deletions could still occur. Although these aforementioned measures improved the identification of a recombinant BACMID, the system was however still very unstable and inefficient.

Recombinant baculovirus DNA was prepared by transposition of the VP2 and VP5 genes into the BACMID shuttle vector. White colonies representing recombinant BACMID DNA were selected. Recombinant BACMID DNA was isolated and transfected into Sf-9 insect cells. Recombinant baculovirus stock was harvested 72 hours after transfection. Recombinant baculoviruses containing the VP2 gene, the VP5 gene and both VP2 and VP5 genes were generated in this way.

4.3.6 mRNA detection of VP2 and VP5

The transcription of VP2 and VP5 genes in recombinant baculovirus infected insect cells was determined by mRNA dot blots. Insect cells were infected with recombinant baculoviruses containing the VP2 gene and the VP5 gene respectively and a recombinant with both the VP2 and VP5 genes. The cells were harvested 36 hours after infection and blotted in duplicate onto a nitrocellulose membrane. For the detection of VP2 and VP5 specific mRNA duplicate membranes were hybridised with AHSV-3 VP2 and AHSV-3 VP5 specific ^{32}P labeled probes. After hybridisation the membranes were washed, dried and autoradiographed.

The mRNA dot blot is shown in Figure 4.8. The mRNA of insect cells blotted in row (a) was hybridised with the VP2 specific probe and in row (b) with the VP5 specific probe. Neither the VP2 of the VP5 probe hybridised to the mRNA of the mock infected

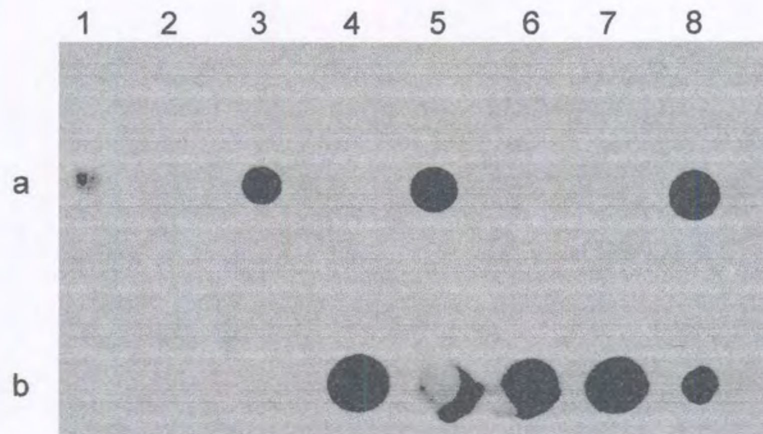

Figure 4.8: Autoradiograph of mRNA dot blots of dual probed with (a)a VP2 specific probe and (b) VP5 specific probe. The mRNA was, as indicated below, obtained from Sf-9 cells infected with different baculovirus recombinants or appropriate controls.

Lane 1: Mock infected Sf-9 insect cells.

Lane 2: Wild type baculovirus

Lane 3: Recombinant baculovirus with VP2 cloned under the polyhedrin promoter of pFastbac vector

Lane 4: Recombinant baculovirus with VP5 cloned under the polyhedrin promoter of pFastbac vector

Lane 5: Recombinant baculovirus with VP2 cloned under the polyhedrin promoter of pFastbac Dual vector

Lane 6: Recombinant baculovirus with VP5 cloned under the p10 promoter of pFastbac Dual vector (clone 1)

Lane 7: Recombinant baculovirus with VP5 cloned under the p10 promoter of pFastbac Dual vector (clone 2)

Lane 8: Recombinant baculovirus with VP2 cloned under the polyhedrin and VP5 cloned under the p10 promoter of pFastbac Dual vector infected cells.

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and wild type baculovirus infected insect cells (lanes 1 and 2). Lanes 3 and 4 demonstrate the specificity of the probes. The VP2 and VP5 probes hybridised respectively to the insect cells infected with either AHSV-3 VP2 (row (a),lane 3) or AHSV-3 VP5 (row (b) lane 4) recombinant baculovirus. Hybridisation of the VP2 probe to row (a), lane 5 shows mRNA transcription of the VP2 gene cloned under the polyhedrin promoter of the pFastbac DUAL™ vector. Similarly transcription of the VP5 gene cloned under the p10 promoter of the pFastbac DUAL™ vector is shown in row (b), lanes 6 and 7 by hybridisation to the VP5 probe. Hybridisation of lane 5, row (b) may be the result of an overflow of cells from lane 6. The hybridisation both VP2 and VP5 probes to lane 8 representing the double recombinant indicated the successful transcription of both the VP2 and VP5 genes in recombinant baculovirus infected insect cells.

4.3.7 Co-expression of AHSV-3 VP2 and VP5

Recombinant baculovirus expressed proteins were radiolabeled to determine if AHSV-3 VP2 and VP5 genes were not only transcribed into mRNA but also translated in insect cells. Insect cells were infected with recombinant baculoviruses containing the VP2 and VP5 genes as described in the legend of Figure 4.9. The proteins were pulse labeled with ^{35}S methionine for four hours from 36 hours after and analysed by 15% SDS PAGE and autoradiography (Figure 4.9). VP2 can be seen as a unique band in lanes 3, 5 and 7 corresponding to the VP2 protein of the *in vitro* translated protein control. VP5 can be seen as a unique band in lane 4 also corresponding to the VP5 positive control (lane 8). The absence of the VP5 in (lane 6) may be the result of poor translation of the mRNA previously shown to be present and is therefore not detected. In lane 5 the dual expressed VP2 and VP5 (Figure 4.7: row (b), lane 8) proteins are shown. The VP2 is a unique band corresponding to VP2 in lanes 3 and 8. In the position of VP5 a very faintly labeled band is present. The band could be recombinant expressed VP5. The amount produced is however so small that it is barely detectable. The poor expression of the VP5 appears to confirm lower level VP5 mRNA transcription as seen in the results shown in Figure 4.8.

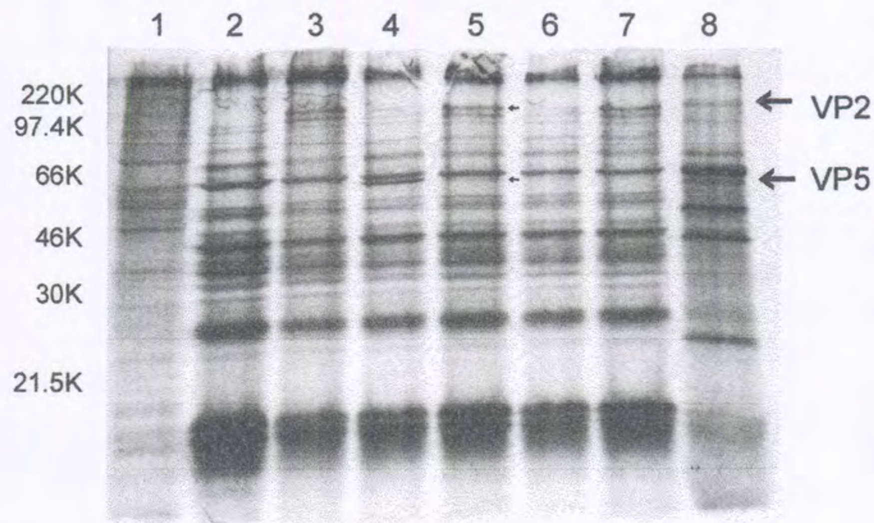

Figure 4.9: Autoradiograph of 15% SDS PAGE of proteins labeled in Sf-9 cells 36hpi infected with different recombinant baculoviruses as indicated in lanes 1-8. The VP2 and VP5 genes are respectively cloned under the polyhedrin promoter of the pFastbac vector. For the dual expression of AHSV-3 VP2 and VP5, the VP2 gene is cloned under the polyhedrin promoter and the VP5 gene is cloned under the p10 promoter of the pFastbac DUAL vector.

Lane 1: Mock infected
Lane 2: Wild type baculovirus insect cells.
Lane 3: AHSV-3 VP2 (pFastbac) recombinant baculovirus
Lane 4: AHSV-3 VP5 (pFastbac) recombinant baculovirus
Lane 5: AHSV-3 VP2 and VP5 (pFastbac Dual) recombinant baculovirus
Lane 6: AHSV-3 VP5 clone 1 (pFastbac Dual) recombinant
Lane 7: AHSV-3 VP5 clone 2 (pFastbac Dual)recombinant
Lane 8: In vitro translated AHSV ds RNA

4.4 Discussion

As discussed in the introduction, there are indications that VP5 may play an important role in interacting with VP2 to present serotype specific antigens more effectively (Martinez-Torrecuadrada *et al.*, 1996, Martinez-Torrecuadrada *et al.*, 1994, Mertens *et al.*, 1989). An attempt to express AHSV-3 VP5 by itself or together with VP2 in insect cells using the Bac-to-Bac™ expression system was made. The successful co-expression of VP2 and VP5 by means of the pFastbac DUAL™ vector could form the basis of further investigations in VP2- VP5 interactions as well as a basis for the formation of AHSV VLP's.

The AHSV-3 VP5 PCR gene product that was provided by Frank Vreede (OVI) was cloned into an *in vitro* expression system and transcribed and translated *in vitro.* A band corresponding to the VP5 specific band of the *in vitro* translated VP5 indicated that the VP5 protein was indeed successfully transcribed and translated from the PCR copy of the VP5 gene. However a disturbing result was that a large amount of unique smaller peptides were also translated. One possible explanation is the large number of ORF on the AHSV-3 VP5 gene (Figure 4.2). All these ORF's could potentially encode a protein of a specific size. Many of the smaller or 'truncated' VP5 protein seen in lane 5 of Figure 4.1 correspond to predicted sizes of proteins encoded form internal ORF's (Figure 4.2). In this figure the positions of the start and stop codons of each open reading frame (ORF) are indicated. The predicted protein size is given next to each ORF. Also shown is the flanking Kozak sequence of each initiation codon. The Kozak sequences are important ribosome recognition sites (Kozak, 1982, Kozak & Shatkin, 1979). The longest ORF with a start codon is flanked by the predicted Kozak sequence and encodes full-length VP5 protein. Of all the identified internal start codons three, in positions 107, 461 and 914, encoding for a 53K, a 40.5 K and a 21K protein respectively are flanked by Kozak sequences. Most of the other start codons contain either the -3 purine or the G^{+4} or no Kozak sequence at all. The 40.5 K and 21K truncated proteins are represented by relatively strong bands. The 21K band is however very diffuse. This may be as a result of the 12% protein gel which does not separate small peptides effectively. A possible explanation for this phenomenon may

be that *in vitro* translation systems, especially the Rabbit reticulocyte lysate translation system recognized internal AUG as start codons (Kozak, 1989). In the literature truncated AHSV VP5 protein of approximately 50K have been reported (Grubman & Lewis, 1992, Martinez-Torrecuadrada *et al.*, 1994). The many internal start codons may be significant. The AHSV NS3 gene has two in phase start codons that encodes the NS3 and NS3A proteins (van Staden & Huismans, 1991). In other organisms such as picornaviral RNA (Gunnery *et al.*, 1997) and GCN4 yeast (Vilela *et al.*, 1998) multiple ORF's have been indicated to play a role in transcriptional control of the protein. It may be important to determine if internal start codons play a role in translational control of AHSV VP5 gene .

The AHSV-3 VP5 gene was cloned into the pFastbac transfer vector, transposed into the BACMID genome to produce AHSV-3 VP5 specific baculoviruses. VP5 specific proteins were expressed in insect cells infected with the recombinant baculovirus. AHSV-3 VP5 specific mRNA was detected in infected insect cells confirming transcription of the gene. A 56.4K protein of the expected size of VP5 (Roy *et al.*, 1994b) was seen as a unique band on a 15% SDS PAGE gel. This result was confirmed by pulse labeling of proteins with ^{35}S methionine. These results corresponded with the expression of AHSV-4 VP5 protein as baculovirus recombinant proteins (Martinez-Torrecuadrada *et al.*, 1994). The expression of the AHSV-3 VP5 protein is much higher than the reported expression of AHSV-9 VP5 (du Plessis, 1995). The AHSV-3 VP5 protein was expressed successfully in insect cells with the use of the Bac-to-Bac^{TM} baculovirus expression system. Dual expression of the AHSV-3 VP2 and VP5 proteins, using the new pFastbac DUAL vector was subsequently attempted.

The VP2 gene was cloned under the polyhedrin promoter and the VP5 gene under the p10 promoter of the pFastbac dual transfer vector of the Bac-to-Bac^{TM} expression system. To determine if the polyhedrin and p10 promoters were functional the genes were initially cloned separately under the respective promoters. Once effective mRNA transcription of the genes was confirmed by mRNA dot blot, the VP5 gene was cloned under the p10 promoter of to the pFastbac Dual transfer vector already containing the VP2 gene under the polyhedrin promoter. This process was

hampered by the instability of the dual transfer vector. Many random deletions made the screening on a large number of recombinants necessary in order to find a recombinant transfer vector containing both genes and all the necessary information needed for effective transposition into the BACMID genome. The same problem was also experienced by others expressing different genes using the Bac-to-Bac™ expression system.

The transcription of the genes in insect cells was detected by a mRNA blot. Successful mRNA transcription of both the VP2 and VP5 genes was shown. However the VP5 specific mRNA transcribed *in vivo* from the dual expression vector concentration was very low. This is also evident from the expression levels of the corresponding proteins VP2 and VP5 as was shown by pulse labeling of the proteins with ^{35}S methionine. VP2 was successfully expressed while the VP5 protein levels were very low. This is the first successful dual expression of AHSV-3 VP2 and VP5 proteins of AHSV using the Bac-to-Bac baculovirus expression system. Other than AHSV-4 VP2 and VP5 dual expression (Martinez-Torrecuadrada *et al.*, 1994), no other AHSV outer capsid proteins have been expressed together. Similar results were observed with the dual expression of AHSV-4 VP2 and VP5. The expression levels of both proteins were very low. It has been suggested that the proteins may be toxic due to the observation that cells infected with baculovirus recombinants that express both VP2 and VP5 show earlier signs of cell death than cells infected by wild type baculovirus. This may have resulted the in low VP2 and VP5 expression levels (Martinez-Torrecuadrada *et al.*, 1994). However no premature cell death was observed during infection of insect cells with recombinant baculovirus containing both the VP2 and VP5 genes of AHSV-3. Other than for serotype 4 and now serotype 3 no successful dual expression of AHSV VP2 and VP5 proteins has been reported. The dual expression of serotype 9 VP2 and VP5 was attempted. Indications are that mRNA of both genes was transcribed but only the expression of VP5 was shown (du Plessis, 1995). Two types of baculovirus dual expression systems have been attempted. The pAcUW3 was used for expressing VP2 and VP5 of AHSV serotypes 4 (Martinez-Torrecuadrada *et al.*, 1994) and serotype 9 (du Plessis, 1995). The Bac-to-Bac™ expression system with the use of the pFastbac DUAL™ transfer vector was used for

the dual expression of AHSV-3 VP2 and VP5 also with unsatisfactory results especially with regard to the VP5 expression levels. Unfortunately due to the extremely poor VP5 expression levels no further assays on the effect of dual expression of AHSV-3 VP2 and VP5 genes on the solubility of the VP2 were carried out.

The Bac-to-Bac™ dual expression system initially seemed to be a very convenient system for the simultaneous expression of two proteins. However many unexpected problems were experienced in the construction of the dual recombinant baculovirus. The instability of the pFastbac Dual transfer vector together with the low expression levels of the proteins has to be carefully evaluated to determine if the results justify the time and effort invested to gain a stable recombinant for dual expression. In this case the results were very unsatisfactory because the initial question of whether the VP2 solubility was influenced by co-expression with the VP5 protein remained unanswered due to the poor expression levels that were achieved. The alternative expression system used did not solve the problem that was previously experienced with the low levels of VP5 expression. It would appear as if the expression level of VP5 is affected by factors that are totally independent of the expression vector. It is possible that the number of internal start codons identified in the VP5 gene may play a role in controlling the levels of protein expression. Further investigation is however necessary to determine this is indeed the case.

CHAPTER 5

Concluding remarks

The outer capsid of the AHS virion consists of the major structural proteins VP2 and VP5 encoded by the dsRNA genome segments 2 and 6 respectively (Roy *et al.*, 1994b). The VP2 protein has been identified as the major serotype specific antigen and is therefore a candidate for a subunit vaccine against AHSV infection. This has been confirmed by the successful protection against a challenge with virulent AHSV-4 after induction of a protective neutralising immune response in horses by immunisation with recombinant baculovirus expressed AHSV-4 VP2 (Roy *et al.*, 1996). This success has motivated projects to clone and express all VP2 proteins representing the nine serotypes in order to develop a VP2 based subunit vaccine protecting against all AHSV serotypes.

In the Genetics department at the University of Pretoria the AHSV-3 VP2 gene was cloned, sequenced and expressed as recombinant baculovirus protein (Vreede & Huismans, 1994). In an successful attempt to increase the expression level of the AHSV-3 VP2 protein the gene was recloned into the Bac-to-Bac™ expression system by Grant Napier and a very high expression level was observed. Recombinant AHSV-3 VP2 expressed with the Bac-to-Bac™ however elicited only a very poor immune response (personal communication). It was determined that the majority of the recombinant baculovirus expressed VP2 protein was found in an aggregated form in insect cells. Possible cause of the aggregation of AHSV-3 VP2 was investigated. Thereafter the primary focus of the investigation was focused on increasing the solubility of baculovirus expressed VP2 protein. Two approaches were investigated. Firstly, the possibility to either chemically denature and refold the recombinant protein. Secondly, the co-expression AHSV-3 VP2 with AHSV-3 VP5 in order to improve the solubility and therefore antigenicity of the AHSV-3 VP2 recombinant protein as has been reported in the case of BTV. The AHSV-3 VP5 gene has not previously been characterised. The nucleotide sequence of the gene was therefore determined and the protein expressed in order to determine its suitability for dual expression with

AHSV-3 VP2 as baculovirus recombinant proteins using the Bac-to-Bac™ expression system.

It was found that the recombinant AHSV-3 VP2 protein was predominantly in a particulate form in insect cells. Only a small amount of soluble protein could be observed during early stages of infection. The aggregation was most probably due to the high concentration of VP2 in the infected Sf-9 insect cell. Other factors such as temperatures below freezing caused any soluble VP2 protein to aggregate. Detergent concentrations of higher than 0.1% also rendered soluble protein insoluble. In order to harvest a small amount soluble protein it is therefore recommended to harvest not later that 42hpi. Freezing of the cells is not recommended. Cell lysis with buffers containing detergents in concentrations higher than 0.1% may also cause aggregation of the soluble VP2 fraction.

It was observed that most of the recombinant AHSV-3 VP2 protein was lost due to aggregation. De-aggregation of particulate VP2 protein with 6M Guanidinium HCl was quite successful. However attempts to refold the protein using size exclusion chromatography for the removal of the denaturing salt resulted in a large loss of VP2. The removal of the Guanidinium HCl from the VP2 protein is very important due to the highly toxic nature of the Guanidinium HCl. Although a small amount of 'refolded' salt free protein was obtained, the process was very inefficient and difficult to reproduce on a large scale. The process is also very time consuming and expensive. These are all factors which are not desirable for vaccine development which requires a reproducible and cost-effective procedure for the preparation of the subunit vaccine. A biological approach in solving the solubility problem was therefore investigated.

An attempt was made to co-express the AHSV-3 VP2 and VP5 proteins and by this approach increase the solubility and immunogenicity of VP2 through possible VP2-VP5 interaction. The AHSV-3 VP5 gene was first characterised and determined to be 1566pb in length encoding for a 505 amino acid protein of approximately 56K. For the first time the sequence of more than two AHSV VP5 genes were compared. Very interesting regions were identified of which two could possibly play a role in the

serotype or serogroup specificty of the AHSV VP5 protein or interaction of the VP5 protein with VP2 or VP7. These regions, however need to be further investigated to ascertain their importance. Furthermore highly conserved nucleotide sequences in the 5' and 3' coding regions were identified. These regions could possibly play a role in regulation of translation of the VP5 protein as well as virus assembly. mRNA secondary structure modeling, together with mutational analysis, could possibly target important regions of secondary structure within these conserved nucleotides.

The AHSV-3 VP5 protein was successfully expressed as a baculovirus recombinant. For the first time AHSV VP5 recombinant protein was observed by Coomassie blue staining. The band however was very difficult to detect because of a wild type baculovirus band of similar size. Radiolabeling however confirmed the successful expression of the AHSV VP5 protein. Dual expression of AHSV-3 VP2 and VP5 was subsequently attempted. VP2 and VP5 were co-expressed unfortunately the expression level of VP5 very low. This was a disappointing result because the co-expression of the AHSV VP2 and VP5 was intended to be used as a tool to determine if the AHSV VP5 protein influenced the solubility of the VP2 protein. These studies, however had to be abandoned due to the low levels of expression of the proteins, especially the VP5 protein.

Many problems were experienced with the instability of these dual expression vector resulting in a very inefficient and long cloning procedure. A large number of recombinants had to be screened in order to select a clone which contained both genes and all the other important sections of the vector for successful expression of the VP2 and VP5 proteins. The dual expression of AHSV VP2 and VP5 seems to be problematic irrespective of the expression system used. AHSV-4 VP2 and VP5 were successfully expressed but also at very low expression levels (Martinez-Torrecuadrada *et al.*, 1994). AHSV-9 VP2 and VP5 co-expression was attempted. mRNA transcription of both genes was shown but no dual expressed proteins were shown (du Plessis, 1995). This may be due to a factor other than the expression system. A possible explanation may be the numerous internal start codons identified on the VP5 gene of

serotype 3. These start codons may play a role in translational control and result in low expression levels of VP5.

Another, but less likely explanation may be PCR induced mistakes in the AHSV VP5 gene. A cDNA/ PCR hybrid to replace the largest part of the gene with the original cDNA may be a strategy to eliminate possible mistakes in the gene. Another alternative would have been to sequence the AHSV PCR product and compare the sequence of the PCR product to the determined cDNA sequence.

The AHSV-3 VP2 induces a poor neutralising immune response when the protein is aggregated. The chemical de-aggregation followed by a refolding process and the dual expression of the proteins were ineffective. For the use of AHSV VP2 as subunit vaccine, the protein expression levels and the serotype specific antigenicity of VP2 are important. Neither the chemical solublilisation nor the dual expression of VP2 with VP5 succeeded to meet these demands. Alternative means of presenting neutralising epitopes of AHSV VP2 will therefore have to be developed.

The identification of peptides containing serotype specific, neutralising epitopes seems to be a strategy that may provide better results. In the Genetics department at the University of Pretoria two projects were launched in order to address these approaches. Truncated AHSV-9 VP2 proteins were cloned, expressed as baculovirus recombinants and immunogenic peptides were identified. A parallel project on AHSV-3 VP2 was based on the creation of a phage display library of the AHSV-3 VP2 in order to identify neutralising epitopes. The peptides on which these epitopes are located could be presented as part of these bacteriophages and developed as potential subunit vaccines for protection against AHSV infection.

The sequence data of characterised AHSV VP5 genes could also form a basis for analysis of AHSV VP5 gene regulation. The mRNA appears to be translated very poorly. It is possible that the sequence of the gene may play a role in affecting the low levels of protein expression. Secondary structure of mRNA may play a role in the regulation of expression levels of genes. This could be further investigated by

secondary mRNA modeling and mutagenesis. An understanding of AHSV gene regulation may be gained as well as a possible means of increasing the expression levels of the AHSV VP5 protein by mutation of regulatory regions.

In conclusion: The reason for aggregation of AHSV -3 VP2 in the Sf-9 cells appears to be primarily related to protein concentration in the cell but external factors such as freezing of the cells and detergents could also play a role in the solubility of the AHSV-3 VP2 protein. Both chemical and biological attempts were made to solve the problem of AHSV-3 VP2 aggregation. These results have shown that both chemical de-aggregation and the dual expression of AHSV-3 VP2 and VP5 proteins provided possible solutions to solving AHSV-3 VP2 aggregation in the laboratory. However both strategies were highly inefficient and therefore cannot be used on a large scale for vaccine purposes. Chemical solubilisation of VP2 is inappropriate because it is a labor intensive and time consuming procedure, resulting in poor yields of soluble VP2. The very poor expression levels of VP5 when co-expressed with VP2 prevented the evaluation of effect of VP5, on the solubility of VP2. Alternative ways of developing AHSV specific subunit vaccines such as peptides, nucleic acid vaccines or viral vector vaccines may offer a more reliable and stable system for inducing a protective immune response.

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

Poster:

Napier, G.B., Filter, R.D. and Huismans, H. **High-Level expression of the Outer Capsid Protein of African Horsesickness Virus.** 10th International Congress of Virology Division, International Union of Microbiological Societies. Jerusalem, Israel, August 1996.

Presentation:

Filter,R.D. and Huismans H. **The characterisation of VP2 of AHSV-3 with the aim of developing a serotype specific subunit vaccine.** South African Genetics Society XVIth Congress, Bloemfontein, July 1998

Paper:

Filter, RD; Vreede,F.T. and Huismans. **The characterisation of African Horsesickness virus serotype 3 outer capsid protein VP5.**
To be submitted

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