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THE CLONING AND EXPRESSION OF THE MAJOR CORE PROTEIN, VP3 FROM AHSV-3 AND AHSV-9

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THE CLONING AND EXPRESSION OF THE MAJOR CORE PROTEIN, VP3 FROM AHSV-3 AND AHSV-9

by

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SUMMARY.

An important goal in AHSV biology, is understanding the way in which the virus structural proteins are assembled to produce the mature virion. Work done in several laboratories using the baculovirus expression system to express these structural proteins, alone or in combination, has gone a long way towards elucidating the problem. This strategy has demonstrated the *in vivo* assembly of core-like and virus-like particles which closely resemble the native virus. When the major core proteins VP3 and VP7 of BTV were co-expressed in insect cells using the baculovirus system, the formation of core-like particles resulted. It was therefore of interest to investigate whether this would also occur if the cognate VP3 and VP7 genes of AHSV were expressed using this system. However, in order to initiate this work, the VP3 gene of AHSV first needed to be cloned, and its expression in the baculovirus system tested.

Full length cDNA copies of the VP3 genes of both AHSV-3 and AHSV-9 were cloned. Sequence analysis of their open reading frame termini revealed a similar high degree of conservation as is found amongst the VP3 genes of BTV. Bacterial expression and *in vitro* transcription and translation demonstrated that this gene could be expressed as a full length protein. A baculovirus recombinant of this gene revealed the synthesis of a full length gene product. However, upon production of high titre stocks of the virus, the levels of protein produced declined dramatically. This may have been attributable to an instability in the parental virus.

Subsequent to this study, an alternative system was used to produce a VP3 baculovirus recombinant. This approach significantly improved the yield of recombinant VP3. When this recombinant was expressed together with a AHSV VP7 baculovirus recombinant, core-like particles were produced which closely resembled those seen for BTV (Maree S., Durbach S.I. and Huismans H.[submitted]).

OPSOMMING.

Die samestelling en die struktuur van die orbivirus is deur vorige studies ondersoek deur die ekspressie van die strukturele proteïene in die bakulovirus ekspressie sisteem as enkel monomere of as heterogene kombinasies van die strukturele proteïene. Hierdeur is die *in vivo* samestelling van die kernagtige en virusagtige partikels was 'n nou ooreenkoms getoon het met die natiewe virus gedemonstreer. Ko-ekspressie van die hoop kernproteïene VP3 en VP7 van die BTV orbivirus het gelei tot die onstaan van kernagtige partikels. Dit was dus van belang om ondersoek in te stel om te bepaal of die ekspressie van die ooreenstemmende VP3 en VP7 gene van die AHSV orbivirus in die bakulovirussisteem ook sou lei tot hierdie fenomeen. Die VP3 geen van AHSV moes egter eers gekloneer word en die ekspressie van hierdie proteïen in die bakulovirussisteem bepaal word alvorens hierdie studie begin kon word.

Vollengte cDNA kopïee van die VP3 gene van AHSV-3 en AHSV-9 is gekloneer. DNA volgorde bepaling van die 5' en 3' kante van die oop leesrame van hierdie twee VP3 gene het bewys dat die hoë mate van DNA homologie vergelykbaar is met die tussen die VP3 gene van BTV serotipes. Uitdrukking van die rekombinante VP3 proteïen in 'n prokarioot (*E. coli*) asook *in vitro* transkripsie en translasie het bewys dat hierdie geen as 'n vollengte proteïen uitgedruk kon word in hierdie sisteme. Op dieselfde wyse is aangetoon dat dit ook geld vir die bakulovirus ekspressie sisteem. Daar is egter bevind dat ekspressie van rekombinante proteïen gedaal het na die produksie van hoë titer virus wat moontlik toegeskryf kon word aan 'n inherente onstabiliteit van die oorspronklike virusisolaat.

Opvolgend op hierdie studie is 'n alternatiewe sisteem gebruik om 'n VP3 bakulovirusrekombinant te produseer wat die opbrengs van die rekombinante VP3 proteïen beduidend verhoog het. Ko-ekspressie van hierdie VP3 bakulovirusrekombinant het gelei tot die produksie van kernagtige partikels wat 'n nou ooreenkoms getoon het met die ooreenstemmende BTV partikels [Maree S., Durbach S.I. and Huismans H (voorgelê)].

LIST OF ABBREVIATIONS.

AHSV	African horsesickness virus
BHK	baby hamster kidney
BTV	Bluetongue virus
C	cytosine
cDNA	(complementary) deoxyribonucleic acid
DEPC	diethyl pyrocarbonate
dNTP	2'-deoxyribo-5'-nucleoside triphosphate
dsRNA	(double stranded) ribonucleic acid
DTT	1,4-dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
EHDV	epizootic hemorrhagic disease virus
FCS	foetal calf serum
G	guanine
GST	guanosyl transferase
IPTG	isopropyl-β-D-thiogalactopyranoside
LB	Luria Bertani
MAK	methylated albumen Kieselguhr
MCS	multiple cloning site
NS	non-structural
ORF	open reading frame
OVI	Onderstepoort veterinary institute
PAGE	polyacrylamide gel electrophoresis
pBS	plasmid Bluescribe
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PSB	protein solvent buffer
rNTP	2'-ribonucleoside-5'-triphosphates
SDS	sodium dodecyl sulphate

sf	*Spodoptera frugiperda*
SSC	sodium chloride; trisodium citrate
STE	sodium chloride; tris; EDTA buffer
TAE	tris; acetic acid; EDTA buffer
TE	tris; EDTA buffer
UHQ	ultra high quality
U	units
VP	virus protein

Dedicated to my family and Lena

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To Darryl, for that bit at the end..

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Chapter 1 Literature Review.

1.1 Introduction.

African horsesickness virus (AHSV), the etiological agent of African horsesickness, is an orbivirus and member of the family Reoviridae. The virus is biologically and genetically similar to the well characterized prototype orbivirus, bluetongue virus (BTV). Nine distinct serotypes have been identified. The virus is transmitted by a *cullicoides* vector to horses and may in many cases result in death. The disease, which is primarily distributed throughout sub-saharan Africa (Mellor, 1993) has now, in recent years, been appearing in North Africa and Southern Europe (Mellor, 1993; Zientara *et al*; 1993).

Structurally, AHSV consists of two capsid layers. An outer capsid consisting of the major proteins, VP2 and VP5 which are predominantly involved in cell tropism and cell entry within the eukaryotic host, and aspects of immune recognition. The virus also has an inner capsid called the core, which is composed of the two major proteins VP3 and VP7 and three additional minor proteins. This structure is responsible for transcription of the viral genome, and consequently, the production of progeny virus. Several non-structural proteins are also synthesized during the course of an infection, which are not directly associated with the virion, but are involved in aspects of virion assembly and export from the cell. Enclosed within the inner capsid are ten segments of double stranded RNA, each responsible for encoding a single protein product, except the smallest genome segment which encodes two proteins resulting from a second, in frame initiation codon (Van Staden and Huismans, 1991). For a summary of the different genome segments of AHSV and the proteins they encode, see table 1.

Table 1: AHSV genome segments and their encoded proteins (Vreede and Huismans, 1997).

Genome segment	serotype	segment length	5' UTR[1]	3' UTR	viral protein	protein length	predicted M_r
		(bp)	(bp)	(bp)		(aa)	
1	9	3965	13	34	VP1	1305	150292
2	3	3221	12	35	VP2	1057	123063
3	4	2792	26	48	VP3	905	103269
4	4	1978	11	38	VP4	642	75826
5	9	1566	19	29	VP5	505	56771
6	9	1748	35	66	NS1	548	63377
7	9	1167	17	103	VP7	349	37916
8	9	1166	22	46	NS2	365	41193
9	3	1169	17	42	VP6	369	38464
10	9	756	18	84	NS3 NS3A	217 206	23659 22481

[1] UTR = untranslated region.

Before the advent of modern molecular biological tools, many of the functions of the various genes were elucidated by the use of classical genetic methods which are amenable to the study of the Reoviridae because of it's segmented genome. These studies typically involved the preparation of virus reassortants which were obtained from a mixed infection of two strains of a virus, and then getting a gene segment from one virus reassorting into the other. This way, a mutation could be assigned to a particular genome segment by its reassortment into the other parental virus.

Recently the baculovirus expression system has been used very successfully to elucidate the way in which the major structural proteins of BTV interact to form an intact virion. No such studies have yet been carried out with AHSV and therefore a study was initiated to express the VP3 gene of AHSV by means of a baculovirus expression system in order to ultimately gain insight into the role of VP3 in the assembly of an intact virion. Since VP3 is a major component of the core, this literature survey will focus on investigating the role of VP3 in the viral core. It will also attempt to see the orbivirus core in the context of viral core-like structures in general.

1.2 Virus Structural Proteins.

Viruses represent a class of genetic material able to parasitize a eukaryotic host by manipulating the cellular machinery to aid in their replication. Other classes of genetic material exist which, like viruses increase their copy number independently of that of the host genome. However this class of "selfish" DNA, is confined to the host cell, and consequently there will have been selection for them to have a less dramatic effect on the host phenotype (Orgel and Crick, 1980; Doolittle and Sapienza, 1980). The feature which ultimately distinguishes viruses from the former class, is their unique ability to move between cells and hosts. This property has been achieved primarily by the evolution of a protein capsid. Besides the protection this offers the genome, it has also provided them with many of the characteristics which define them.

Nibert *et al.* (1991) divided the virion into two functional categories: the (i) *delivery system* and (ii) the *payload*. The *delivery system* of the virus being that component responsible for transmitting the virus between cells and hosts, and for orbivirus, this function is predominantly effected by the proteins of the outer capsid. The *payload* of the virion, on the other hand, is the structural component responsible for initiating replication within the target cell (see fig 1.1). The focus of this review will be on the virus *payload*, i.e, the

structural components of the virion responsible for initiating an infection upon a virus entering the host cell. In the case of the orbivirus, the *payload* is the core particle. It is important to note that during the course of an infection, non-structural proteins are synthesized which facilitate the process of progeny viral production, and are therefore an important component of the virus *payload*.

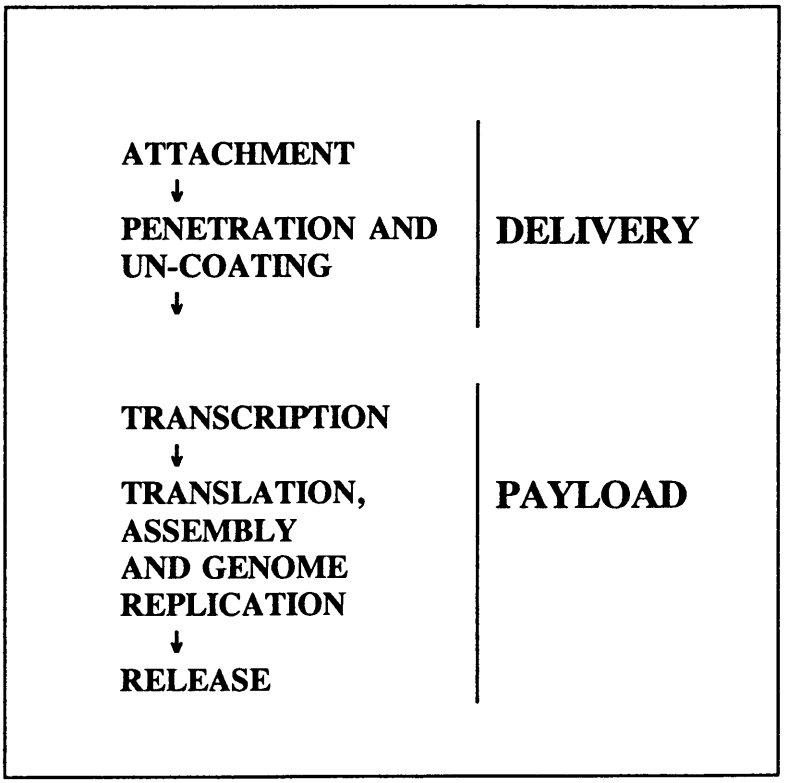

fig. 1.1 **Functional definition of a virus [adapted from Nibert *et al.*, (1991)].**

1.2.1 Brief overview of the orbivirus *delivery system*.

The components of the orbivirus responsible for determining the cell tropism; attachment and entry are located on the distinct outer layer of the orbivirus known as the outer capsid. This layer is composed of the major proteins, VP2 and VP5.

The most surface exposed protein of the orbiviruses is VP2, encoded by genome segment 2. This protein is also the most variable in the orbivirus genus as revealed by comparisons between the capsid protein sequences of different orbiviruses (Iwata *et al.*, 1992), which supports its identification as the serotype specific determinant (Huismans and Erasmus, 1981; Appleton and Letchworth, 1983). This protein, in BTV has the capacity to elicit neutralizing and hemagglutination inhibiting antibodies, and is protective in sheep (Huismans *et al.*, 1987a). Part of this protective response may also be attributed to a serotype specific T-cell response directed against this protein (Takamatsu *et al.*, 1990). A similar situation exists for the AHSV VP2 protein, where neutralizing monoclonal antibodies precipitated VP2 (Burrage *et al.*, 1993). The VP2 protein of AHSV was also able to elicit neutralizing antibodies (Vreede and Huismans, 1994; Martinez-Torrecuadrada *et al.*, 1994) and has been found to be protective in mice (Martinez-Torrecuadrada *et al.*, 1994).

The role of the other major outer capsid protein of the orbiviruses, VP5 has been less clearly defined. It shows approximately the same level of conservation as the group specific antigen, VP7 when compared between orbivirus serogroups (Iwata *et al.*, 1992), i.e approximately 63.4% similar amino acids. It does seem to make some contribution towards serotype determination, as a BTV with a reassorted VP5 induced neutralizing antibodies to its derived serotype (Mertens *et al.*, 1989). Findings that VP5 is partially exposed on the virion surface lend credence to its possible involvement in virus neutralization (Lewis and Grubman, 1990). The protein may also make some contribution to virulence, as was found in one case, where the most obvious difference between a virulent and an avirulent strain of BTV was in the

mobility of the VP5 genome segment (cited in Huismans and Van Dijk, 1990). More recently, reassortant studies with BTV, attributed increased neuro-invasiveness to VP5, an obvious virulence determinant (Carr *et al.*, 1994). Possibly an important role for the VP5 protein is to facilitate the interaction between the highly variable VP2 protein with the more highly constrained structure of the virus core. This has been demonstrated for BTV in the form of virus-like particles, which spontaneously assemble when all four major capsid proteins are simultaneously expressed in insect cells using a baculovirus expression system. In these studies it was found that VP2s from different serotypes could be expressed on the surface of these particles in the presence of VP5 from a single serotype (Loudon *et al.*, 1991).

1.2.2 The *payload.*

The payload is that component of the virus responsible for initiating transcription once inside the host cell, undergoing replication as well release from the cell. There are several reasons for a virus to introduce its genome into the host cell within a relatively complex structure such as a core. Viruses encoding their own transcription/replication machinery seem to require some sort of structural framework with which to initiate transcription. This would increase the local concentration of the required components, which may otherwise be too dilute if they were simply injected into the cell. In the case of the Reoviridae, and other dsRNA viruses, a core structure may also inhibit an interferon response, by keeping the genomic dsRNA separate form the cytoplasm. An interferon response occurs with just minute amounts of dsRNA (Joklik, 1990). A core also provides a scaffold on which to orientate the genome for correct transcription.

In orbiviruses the function of transcription is effected by an intact core particle which is formed after removal of the outer capsid layer of the virus shortly after entry into the cell (Huismans *et al.*, 1987b). The situation is similar in the case of the rotaviruses which also

uncoats upon entry into the host cell. This uncoated single-shelled particle contains the activated viral transcriptase (Bellamy and Both, 1990) which is the functional and morphological equivalent of the BTV core-particle.

Several other groups of viruses which have genomes not directly recognised by the cellular machinery of the host, have core-like structures to which their genomes are attached. The dsRNA containing family of viruses, the Birnaviridae share this characteristic. Transcription also takes place within the single shelled capsid of this virus (Spies *et al.*, 1987). Unlike the reoviridae, however, the virion structure does not need to be altered to expose this activity (Mertens *et al.*, 1982; Spies *et al.*, 1987).

Other viruses, for example those with negative sense RNA as there genomic material, are also dependent on their own transcription/ replication machinery. These viruses also require some form of particle-associated transcriptase. An example of such a virus is the rhabdovirus, which introduce their RNase-protected RNA as a ribonucleoprotein complex with an associated RNA dependent RNA polymerase (reviewed by Banerjee, 1987).

The dsDNA containing poxvirus encode all their machinery required for transcription and replication which takes place in the cytoplasm. However the initial transcription event occurs within the virus core. The proteins resulting, help release the genome from the virus core for subsequent replication (Roizman, 1990).

1.2.2.1 The orbivirus *payload*.

Core Particles

The structure referred to as the *payload* is the core particle in the orbiviruses. This structure is characterized by the absence of the major outer capsid proteins and displays transcriptase

activity (Martin and Zweerink, 1972; Huismans *et al.*, 1987b). The particle consists of the major proteins VP3 and VP7. There are also 3 minor proteins, VP1, VP4 and VP6. These all encircle the 10 segments of dsRNA. It is interesting to note, that the definition of the core-particle as the virus *payload* cannot be simply extended to the virus' life cycle in insect cells, since in that environment the core-particle has been found to be infectious (Mertens *et al.*, 1996).

Besides the structural components directly associated with the virus core, there are several non-structural proteins synthesized during the course of an infection which are intricately involve in the functions of assembly and release of the virus.

The non-structural proteins.

During the early stages of progeny virus assembly, the mRNA is condensed onto structures known as virus inclusion bodies (VIB) prior to being packaged into the developing virion. The non-structural protein NS2 is the major component of these VIBs. Time course studies have demonstrated the development of virions within these structures, which suggests their important role as a site for virus morphogenesis. The presence of virus-specific mRNA has also been located within these structures (reviewed by Gould and Hyatt, 1994). Consistent with its involvement in the condensation and packaging of mRNA, are findings that the protein is capable of binding ssRNA, albeit non-specifically (Huismans *et al.*, 1987c). Interestingly, this binding ability differs between the different orbiviruses (Uitenweerde *et al.*, 1995).

Once the virions have been fully assembled, the structures need to be released from the cell. This is probably achieved with the aid of the smallest and least abundant of the orbivirus non-structural proteins, NS3. This is supported by findings that when NS3 is expressed in the presence of BTV virus like particles, the virus-like structures are exported from the cell

(reviewed by Roy, 1992). Van Staden *et al.* (1995) found clear evidence of a cytotoxic effect of baculovirus expressed AHSV NS3 on insect cells, which they attribute to possible membrane disruption by the protein. A region conserved between different orbiviruses consisting of two hydrophobic domains, was predicted to be a trans-membranous region (Van Staden *et al.*, 1995) lending further support to its being a membrane associated protein and therefore playing a potential role in the release of the virus from the cell.

A feature of orbivirus infected cells is the presence of large tubular structures. These structures have been shown to be composed purely of NS1 (Huismans and Els, 1979) and seem to require no other virus component for their correct assembly as they are evident in insect cells after being expressed as a baculovirus recombinant (Nel and Huismans, 1991; Urakawa and Roy, 1988). The function of these tubules remains largely unknown. The soluble form of the protein may be involved with virion development, as it has been found associated with virus cores and VIBs (Gould and Hyatt, 1994).

The structure of core-particles.

Externally located on the core particles is the major structural protein VP7, which makes up the virus' characteristic capsomeres (Huismans *et al.*, 1987b). VP7 is the most abundant core protein, and has been recognised as a group specific antigen (Huismans and Erasmus, 1981). Studies using monoclonal antibodies directed against certain VP7 epitopes revealed that VP7 does have limited exposure on the surface of the intact virion (Lewis and Grubman, 1990). This exposed region is located at the N-terminal portion of the protein, and is conserved within the serogroup (Li and Yuan Yang, 1990; Wang *et al.*, 1994). VP7 most likely serves as a link between the structurally very conserved inner core, which carries all the transcription machinery, and the outer capsid, which is more subject to genetic change because of exposure to the host immune system.

Beneath the VP7 surface of the core, is a layer of VP3 which is stable, since during the course of an infection, the VP7 component is lost, leaving behind the subcore which is composed of a framework of VP3; the minor core components and the genomic dsRNA (Huismans *et al.*, 1987b). The VP3 scaffold functions to maintain the minor core proteins in the correct conformation to allow transcription to occur. The minor core proteins themselves execute the enzymatic functions required for transcription. The VP3 component of the core will be discussed in greater detail later on in this review.

There are 3 minor core proteins, VP1, VP4 and VP6. The largest orbivirus protein, VP1 is probably the virus encoded replicase-transcriptase based on its primary sequence, which shares limited homology with other virus encoded polymerases (Roy *et al.*, 1994; Vreede and Huismans, 1997). In addition to this, poly (A) synthesis was demonstrated in insect cells, which contained a VP1 baculovirus recombinant, when a poly (A) primer was provided in the presence of a poly (U) template (Roy *et al.*, 1994). The function of capping and methylation of the 5' end of the BTV mRNA, is probably provided by the VP4 minor protein. GTP binding activity has been localised to a baculovirus expressed form of this protein (Roy *et al.*, 1994). The smallest minor core protein, VP6 is probably a helicase based on a conserved motif within the primary sequence of the gene, a property also shared by the AHSV VP6 protein (Turnbull *et al.*, 1996). This protein may therefore be involved in the unwinding of the viral dsRNA prior to the synthesis of the mRNA. Evidence that VP6 is closely associated with the virus genome comes from findings that the protein has strong binding affinity to ssRNA and dsRNA (Roy *et al.*, 1994).

In recent years, a lot of headway has been made concerning the structure and assembly of BTV core particles. This has come about with the expression of the structural components in combinations using the baculovirus expression system. These studies were often done in concert with cryo-electron microscopy, which is an electron microscopy technique which

maintains the 3-D structure of the virus with greater integrity as there is no dehydration and fixing of the sample, only freezing (reviewed by Roy, 1996).

When the major core proteins, VP3 and VP7 were co-expressed in insect cells as baculovirus recombinants, they spontaneously assembled into structures which resembled authentic BTV cores when viewed by negative staining transmission electron microscopy (French and Roy, 1990). Since stable subcores are formed during the course of an infection, consisting of VP3 as the only major protein (Huismans *et al.*, 1987b) it seemed plausible that VP3, if expressed alone could assemble into a structure resembling a subcore. However, when VP3 is expressed with a VP7 which carried a specific point mutation, the assembly of these structures (core-like particles [CLP]) was abrogated, implying that a functional VP7 is required for the synthesis of these structures (LeBlois and Roy, 1993). Subcore-like structures consisting purely of VP3 can be synthesized for BTV, but only by dialysing away the VP7 component with a low salt buffer (Loudon and Roy, 1991). It therefore does seem, that under the conditions used in the baculovirus system, VP7 is required for stable CLP synthesis, and consequently, to provide stability to the virus core. This result is in contrast to that obtained for the tick-borne orbivirus, Broadhaven. In this virus, VP2 (analogue of BTV VP3) subcores could be synthesized by expressing that protein alone using the baculovirus system (Moss and Nutall, 1994). The rotavirus VP3 analogue (called VP2) is also capable of assembling into structures resembling authentic cores without the requirement for VP6 (the BTV VP7 analogue) (Labbé *et al.*, 1991).

The three dimensional structure of BTV cores has been characterised to 30Å resolution by cryo-electron microscopy and image analysis of authentic cores and baculovirus expressed CLPs (Prasad *et al.*, 1992; Hewat *et al.*, 1992). These results have been extensively reviewed (Roy 1992; 1996). The outer most surface of the virus cores consists of 260 trimers of VP7, which are organised into the pentameric and hexaemeric units which make up the virus capsomeres. Located between the VP7 trimers are channels, which in some

cases penetrate through to the inner layer, which is composed of VP3. These channels probably act as a pathway for metabolites, as well as to provide a channel for nascent mRNA. The cryo-electron microscopy work done on the CLP revealed that the VP3 molecules which make up the smooth inner layer of the particle, were roughly disc shaped. Since CLPs are empty, better contrast allowed improved visualization of the structure. The 120 disc-shaped VP3 molecules were arranged as dimers to form the basic icosahedral structure.

The formation of core-particles.

There are two stages during the life cycle of the virus within the mammalian host where core-particles are evident, and consequently, when a transcriptional state is active. The first stage occurs early on during the infection proccess resulting from the uncoating of the parental virus (Huismans *et al.*, 1987b). Later on, the core is also formed as a replicative intermediate during virion assembly as part of the virus' morphogenesis pathway, it is also here that these progeny virus are actively involved in transcription and therefore the production of more virus.

Upon entry into host cells, the initial uncoating event probably occurs in endosomes, and to a lesser extent in lysosomes, which are acidic in nature (Gould and Hyatt, 1994). The acidity is important, as it does have an effect on the concentration of salt required to dissociate VP2 from the virion (Huismans *et al.*, 1987a). Rotaviruses also undergo a similar uncoating event, also at a low pH, to yield single shelled transcriptionally active particles (Bellamy and Both, 1990). For the rotaviruses, the process has been shown to be reversible (Chen and Ramig, 1994a) where the outer coat proteins can be restored *in vitro*, to the single-shelled particles, and are thus capable of recovering their infectivity.

The BTV cores, synthesised as replicative intermediates (Gould and Hyatt, 1994) are found in the virus inclusion bodies (VIB), the sites of virus morphogenesis (Gould *et al.*, 1988) which are composed of the non-structural orbivirus protein, NS2. Similar intermediates may also be found in rotavirus-infected cells (Gallegos and Patton, 1989). Genome replication is most likely to occur at the stage either resembling the subcore particle or at a core particle stage of morphogenesis. However, subcores are considerably less stable than cores (Huismans *et al.*, 1987b). On the other hand, structures above the level of cores, i.e ISVPs (core particles + VP5) and complete virions have no RdRp (RNA dependent RNA polymerase) activity as they are probably inaccessible to the required metabolites. This is supported by the fact that particles containing VP5 and VP2 are unable to undergo transcriptase activity (Van Dijk and Huismans, 1980). Early results of work done on Rotavirus revealed that RdRp activity could occur during assembly at a stage of morphogenesis prior to the addition of any major protein such as the BTV VP3 analogue, VP2. This complex had some minor core proteins present, in addition to several non-structural proteins (Gallegos and Patton, 1989). It was however, recently found that the presence of the major core protein, VP2 may well be necessary. These experiments were done by synthesising the relevant structures using the baculovirus system. They were able to show that the minimum particle supporting replicase activity, was one containing both VP2 and the minor core protein, VP1 (Zeng *et al.*, 1996).

1.2.2.2 VP3, the inner core and its properties.

The protein, VP3 which makes up the scaffold of the inner core, is the most conserved of the major structural proteins of the orbiviruses. Between 57 and 58% of the aligned amino acids between VP3 of BTV and the VP3 of another gnat-transmitted orbivirus, EHDV, are identical to AHSV-4's VP3 (Iwata *et al.*, 1994). This, as compared with VP7, where a similar alignment yielded 44-46% identity and only 19-24% for the outer capsid protein VP2 (Iwata *et al.*, 1994).

This high level of sequence conservation in the VP3 gene may be anticipated due to it's role in maintaining the integrity of the core and some of its functions. The VP3 component of the core has not been shown to have any direct enzymatic role in core functions such as transcription and replication. It's role seems to be mainly structural. It has for example been found that the minor core protein, VP1, interacts directly with the VP3 component of the core. This was proved by co-expressing VP3, VP7 and VP1 in insect cells using the baculovirus system. The resulting CLPs were purified and found to contain, in addition to VP3 and VP7, also VP1. When the VP7 component was removed by dialysis to yield the subcore-like particle, VP1 remained associated with it (Loudon and Roy, 1991).

Much of the sequence conservation is probably due to its interaction with VP7. This can be inferred from experiments where CLP could be synthesized in insect cells, by means of baculovirus expression, using an EHDV VP3 and a BTV VP7, showing that the sites of interaction between VP3 and VP7 have been conserved between the two virus groups (LeBlois *et al.*, 1991). The sites necessary for the association with the other minor core proteins, VP1, VP4 and VP6 are also conserved, as these proteins could also be incorporated into the core-like particles (LeBlois *et al.*, 1991). Attempts to define the regions involved in the formation of core-like particles and the interactions with the minor core proteins were made, by making short deletions in conserved regions of the VP3 molecule, and then assaying for CLP assembly using the baculovirus system (Tanaka and Roy, 1994). Five VP3 mutants were made, and only one, which lacked residues 499 to 508 failed to assemble into CLPs. The critical residues were found, by mutational analysis, to be a methionine residue at position 500 and an arginine at position 502. All those that did form CLPs were also capable of interacting with the minor core proteins. These results suggest that VP3 interactions may, contrary to expectation, be quite flexible. These results are supported by findings that some of the conserved regions of VP3 can be replaced by foreign epitopes, and still assemble into CLPs (Tanaka *et al.*, 1995).

A property of the core attributable to the VP3 component, is its ability to bind ssRNA in a non sequence-specific manner (Loudon and Roy, 1992). It was shown that subcore-like particles derived from baculovirus expressed CLPs, composed purely of VP3, bind ssRNA (Loudon and Roy, 1992). The VP2 protein of Rotavirus (the BTV VP3 analogue) shares this property (Boyle and Holmes, 1986). In addition to this, Boyle and Holmes (1986) showed that the property was independent of the core structure, as it was able to bind the RNA after being transferred onto a membrane after SDS polyacrylamide gel electrophoresis.

There are several stages during the virus' life cycle which may involve an association of ssRNA and the core. Firstly, during the RNA dependent RNA polymerase activity, which resides in the core-particle (Martin and Zweerink, 1972) where, during transcription, ssRNA transcripts are extruded from the particle into the surrounding cytoplasm. The binding of ssRNA to the VP3 may occur during the assembly process, as suggested by Loudon and Roy (1992), where the ssRNA template may bind to the particle, move in and be replicated. This could hypothetically happen synergistically with the non structural protein NS2, which has also been shown to bind ssRNA (Huismans *et al.*, 1987c). Since the ssRNA binds on the outside of the CLP (Loudon and Roy, 1992), it has been suggested that the binding is important in the early stages of morphogenesis. The VP3, for example, may be assembled onto a pre-existing protein/RNA complex. It may also function to orientate the RNA genome correctly for packaging purposes prior to complementary RNA synthesis. In rotavirus, recent work where authentic double layered particles (transcriptionally active analogues of BTV core particles), and synthetic baculovirus expressed VLPs, which were compared by difference imaging of cryo-electron micrographs, showed that the RNA genome forms an icosahedral structure due to its direct association with the rotavirus core (Prasad *et al.*, 1996). The association is with VP2, which gives the core its characteristic icosahedral structure.

VP3 is not the only structural protein with the capacity to bind ssRNA. VP6, a minor core protein, also shares this property. Being a minor protein in possession of helicase-like

motifs, it is probably a component of the RNA polymerase complex. This protein is also tightly associated with the genome, and may therefore have a role during the encapsidation process (Reviewed by Roy, 1992).

1.3 Aims of this Study.

Studies of orbivirus proteins have made considerable progress. This work has been greatly facilitated by the use of the baculovirus expression system (for reviews see Roy, 1992; 1996). Through its use, much has been learned about the order of assembly of orbivirus structural proteins during morphogenesis.

Only recently has this technology been applied to AHSV, and in terms of the structural proteins, both VP2 and VP7 have been successfully cloned, and expressed as baculovirus recombinants (Vreede and Huismans, 1994; Chuma *et al.*, 1992). However, as of yet, VP3 of AHSV has not been expressed in this system. VP3 of serotype 4 has been cloned and shown by sequencing to conform to being the most conserved of the orbivirus structural proteins (Iwata *et al.*, 1994).

The main purpose of this study, was therefore to establish whether VP3 of AHSV could be expressed as a baculovirus recombinant, as was successfully achieved for BTV VP3 (Inumaru *et al.*, 1987). This will allow us the opportunity to investigate whether this protein shares similar properties to its extensively studied BTV counterpart. Not only will this contribute to our basic knowledge of orbivirus biology, but it will also facilitate the development of vaccines in the form of VLP, where VP3, along with VP7 provides the necessary structural framework in the form of CLPs, on which VP2 and VP5 can be presented (Roy, 1992). There is also a possibility, that the CLP itself could contribute to a cell-based immune response (Van Dijk, 1993).

In order to achieve this aim, the VP3 gene of AHSV must first be cloned via a cDNA intermediate, as a full length product, so that it can be tailored for expression in the baculovirus system.

In addition to expressing the gene, it will also be useful to obtain some additional AHSV VP3 sequence information to confirm that this gene is conserved within the serogroup.

Chapter 2. CLONING OF THE VP3 GENES OF AHSV-3 AND AHSV-9.

2.1 Introduction.

The ability to characterise BTV gene function has been greatly facilitated by being able to first clone the gene.

Much of the earlier work in characterising Reoviridae gene function, has relied on the selection of natural mutants. An example of this has been in the characterization of epitopes of VP2 (the BTV protective antigen) important in immune recognition. This work relies on the development of monoclonal antibodies which have the capacity to neutralise BTV infection, and then selecting variants which escape neutralization (De Maula *et al.*, 1993). In this way, regions of VP2 that have a role in neutralization, could be defined. This approach has also proved useful in defining regions of the viral capsid involved in reovirus pathogenesis (Tyler *et al.*, 1993).

Due to the segmented and essentially, monocistronic nature of the orbivirus genome, genome segment reassortment has been used as a tool to analyze the gene function of individual segments. In this way, Mertens *et al.* (1989) showed that VP5 has some role in determining virus serotype when they selected a VP5 reassortant which was able to cross-neutralise with it's parental strain as well as the strain to which it was a reassortant of. Following a similar approach, VP5 was found to have a role in the neuro-invasiveness of newborn mice (Carr *et al.*, 1994). The use of reassortants has also enabled the designation, in temperature-sensitive mutants, of the lesion to their derived genome segment. This, for example has contributed towards the identification of sites important for virion assembly in the outer capsid proteins of rotavirus (Mansell *et al.*, 1994).

The importance of these approaches to characterise gene function cannot be underestimated, as it allows the mutant form of the gene to be studied within the context of the intact virus. However, the mutations studied are serendipitous and rely on specialised selection strategies.

The cloning of Reoviridae dsRNA genome segments was first described for reovirus (Cashdollar *et al.*, 1982). This approach has been successfully applied to other members of the reoviridae, including the orbivirus. The cloning strategy can be briefly summarised as shown in fig. 2.1:

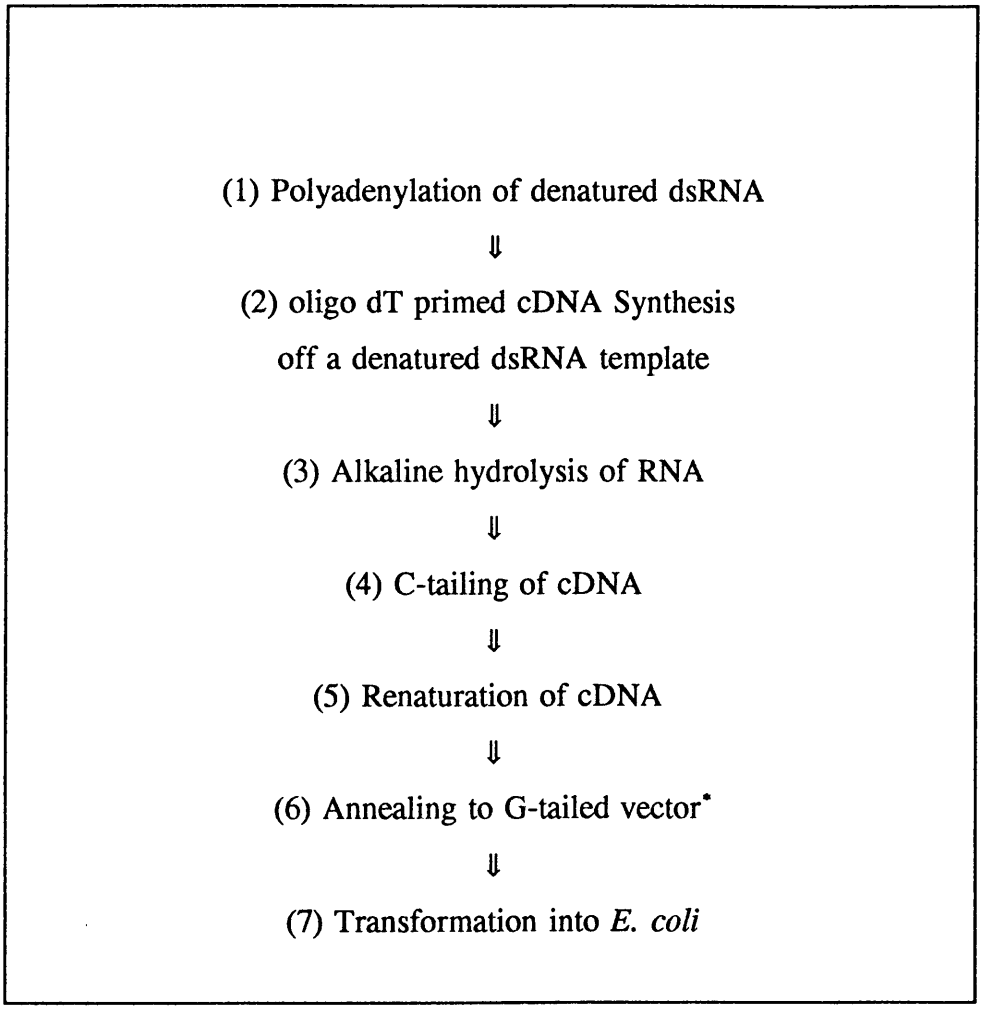

fig. 2.1 Strategy for the cloning of dsRNA genome segments.

The strategy as outlined in figure 2.1 is essentially that described by Cashdollar *et al.* (1982). The technique has in subsequent years, been slightly modified by Huismans and Cloete (1987), where they introduced a sucrose gradient approach to size fractionate the cDNA prior to tailing (step 4), where the approach by Cashdollar *et al.*, (1982), had been to fractionate the cDNA by a gel based method. They also combined steps (5) and (6) as outlined above. An approach which in their hands gave better yields of recombinants.

The cloning of these genome segments has provided a powerful tool for defining functional regions of a gene. In the case of the orbivirus structural proteins, specific regions on VP3 and VP7 required for CLP assembly could be narrowly defined by introducing specific amino acid changes into the cloned sequences of these genes. Co-expressed baculovirus recombinants of these genes were then assayed for their ability to assemble into CLP in insect cells (LeBlois and Roy, 1993; Tanaka and Roy, 1994).

The roles of the structural proteins have also been more clearly defined with respect to the order and nature of their assembly. This has been achieved by expressing baculovirus recombinants of these genes in different combinations and observing their interactions. This has been a successful approach in orbivirus (for reviews, see Roy, 1992; 1996), as well as rotavirus and reovirus (Labbé *et al.*, 1991; Xu *et al.*, 1993).

These studies highlight the advantage of having cloned copies of orbivirus genes as a strategy for studying their functions. Due to the biological similarities of the AHSV serogroup with the prototype orbivirus, BTV in which most of these studies were done, it seems feasible to follow a similar approach to study the function and assembly of AHSV structural proteins.

The BTV segment 3 gene was first cloned by Purdy *et al.* (1984) by the approach developed by Cashdollar *et al.* (1983). Similarly, the AHSV-4 segment 3 gene has also been cloned (Iwata *et al.*, 1994). In a review by Roy *et al.* (1994), it was claimed that the full length

VP3 gene of AHSV was expressed as a baculovirus recombinant, but this has not been unequivocally demonstrated. It was therefore, an aim of this study to clone the 2.8Kb segment 3 gene of AHSV to have it in an accessible form where it could ultimately be expressed as a baculovirus recombinant.

Due to the internal location of VP3, and its interaction with VP7 (French and Roy, 1990), and with at least one of the minor core proteins (Loudon and Roy, 1991), as well as with the dsRNA genome (Loudon and Roy, 1992), it may be expected that this protein is highly conserved. Sequence comparisons between the orbivirus structural proteins has revealed VP3 to be the most conserved major structural protein (Iwata *et al.*, 1994). This high level of conservation is reflected within the BTV serogroup, where comparisons between BTV-10 and BTV-17, have shown the homology at the amino acid level to be 99% (Ghiasi *et al.*, 1985). A similar situation exists within the EHDV serogroup where the VP3 amino acid conservation between EHDV-1 and EHDV-2 is 94.7% (Wilson, 1991). These high levels of conservation will probably also be prevalent in the AHSV serogroup as has already been suggested by hybridization studies (Bremer *et al.*, 1990), but this has not been confirmed by direct sequencing.

The aim of the work undertaken in this chapter, was to obtain a full length cloned copy of the AHSV segment 3 gene, and to sequence the gene partially to investigate whether the high levels of sequence conservation that is found in other orbivirus serogroups is also prevalent within the AHSV serogroup.

2.2 Materials and methods.

2.2.1 Purification and polyadenylation of dsRNA.

dsRNA was purified as previously described (Huismans and Cloete, 1987), and fractionated into it's various size classes (small, medium and large[1]) either by MAK (methylated albumin Kieselguhr) chromatography (Huismans and Cloete, 1987), or sucrose gradient sedimentation (Bremer *et al.*, 1990). The dsRNA was then resuspended in DEPC (diethyl pyrocarbonate) treated UHQ (ultra high quality) water, and stored at -20°C until required.

The polyadenylation was carried out essentially as described by Huismans and Cloete (1987), which is briefly as follows: 10μl of H^3-dATP was freeze dried in an eppendorff tube. To this, 15μl of 1M tris (pH 8.0); 3μl 1M $MgCl_2$; 3μl 0.25M $MnCl_2$; 19μl 4M NaCl and 15μl 5mM dATP were added. 30-40μg of the dsRNA was added, which was either denatured by a 5 minute incubation at room temperature in the presence of 10mM methyl mercuric hydroxide, or alternatively, left undenatured. After diluting the reaction mixture to 0.3ml, 10U of *E. coli* poly-A-polymerase (BRL) was added. The reaction was carried out at 37°C for 15 minutes and then terminated by adding 5μl of 0.5M EDTA (pH 8.0). The unincorporated nucleotides were separated from the poly-A tailed RNA by G-75 sephadex column chromatography. Samples of the eluted fractions, were analyzed in a scintillation counter (Beckman) by mixing 2μl of the sample with 2ml Serenkov scintillation cocktail (Beckman) fluid and then counting for 5 minutes per sample. The fractions containing the polyadenylated dsRNA were pooled and lyophilised and finally resuspended in DEPC treated UHQ water.

2.2.2 cDNA synthesis of polyadenylated dsRNA template.

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[1] small: enriched in segments 7 - 10; medium: enriched in segments 4 - 6; large: enriched in segments 1 - 3.

cDNA synthesis was carried out as described by Huismans and Cloete (1987). For a small scale reaction, approximately 1-2μg of polyadenylated dsRNA was denatured for 15 minutes at room temperature in the presence of 10mM methyl mercuric hydroxide. To this 0.05M Tris (pH 8.3); 0.01M $MgCl_2$; 0.07M KCl; 0.5mM each dNTP; 3mM β-mercapto-ethanol; 60U Human Placental Ribonuclease inhibitor (Amersham); 20μCi [α-^{32}P] dCTP and 200U of Moloney Murine Leukaemia virus RNase H^- Reverse Transcriptase (BRL), were added. The reaction was incubated for 1 hour at 42°C. The unincorporated nucleotides were then separated from the cDNA by G-75 column chromatography. The eluted samples were directly analyzed by scintillation counting. The fractions containing the cDNA were pooled and lyophilised. The freeze-dried cDNA was resuspended in 10x alkali buffer (0.3M NaOH; 20mM EDTA) and analyzed by alkaline agarose gel electrophoresis. The electrophoresis buffer was composed of 1xTAE [0.04M Tris (pH7.4); 1mM EDTA (pH 8.0); 0.03M NaOH and 2mM EDTA). If the cDNA was deemed satisfactory, then the reaction was appropriately scaled up.

2.2.3 Purification and size fractionation of cDNA.

Two approaches were followed to fractionate the cDNA into different size classes. i) Fractionation by alkaline sucrose gradient sedimentation and ii) by purifying the cDNA separated by alkaline agarose gel electrophoresis.

Purification and size fractionation of cDNA by alkaline sucrose gradient sedimentation.

The fractionation of the cDNA by alkaline sucrose gradient sedimentation was done as described by Huismans and Cloete (1987) and is briefly as follows: A 5 times scaled up cDNA synthesis reaction which had been lyophilised (as described in section 2.2.2), was resuspended in 0.1ml 10x alkaline buffer for the RNA hydrolysis. This was left to stand for

10 minutes at room temperature before being layered onto a 10-40% (w/v) discontinuous sucrose gradient made in 1x alkaline buffer (0.03M NaOH; 2mM EDTA). The gradient was centrifuged at 200 000g for 16 hours at 4°C in a SW50 rotor. The gradient was collected in 8 drops per fraction, and the different samples analyzed directly by scintillation counting. Samples containing the cDNA were further analyzed on a vertical 2% (w/v) agarose gel composed of 1x alkaline buffer. The gel was subsequently dried and then autoradiographed overnight at -70°C. The desired fractions of cDNA, identified from the autoradiographed gel, were then purified from the gradient eluate using Geneclean™ (BIO 101). The procedure was performed by first neutralizing the pH of the eluate by the addition of 100x TE buffer (1M Tris [pH 7.4]; 100mM EDTA). Two volumes of NaI was added to the eluates together with 10μl of glassmilk slurry supplied in the Geneclean™ kit. The binding step was carried out at room temperature on an orbital shaker for 1 hour. The mixture was briefly centrifuged and washed 3 times with the NEW wash (supplied in the Geneclean™ kit). After each wash, the suspension was briefly centrifuged to separate the glass matrix from the supernatant. The cDNA was then eluted from the glass matrix in 1M Tris (pH 8.0) at 50°C for 3-5 minutes, and then briefly centrifuged. The elution containing the cDNA was analyzed by scintillation counting of the eluate and glass bead fractions.

Purification and size fractionation of cDNA by alkaline agarose gel electrophoresis.

This method is similar to the method described by Huismans *et al.* (1987). A 5 times scaled up cDNA reaction which had been lyophilised and resuspended in 10x alkaline buffer, was electrophoresed on a vertical 2% (w/v) agarose gel containing 1x alkaline buffer and 1xTAE. After electrophoresis the gel was autoradiographed wet for several hours, and the desired segments excised from the gel after their identification on the autoradiogram. The cDNA was purified by the Geneclean™ procedure, as described previously in this section, except

that the gel slices were warmed to 50°C for 5 minutes upon addition of the NaI to melt the agarose. The cDNA was finally eluted in UHQ water and stored at -20°C.

2.2.4 C-tailing of cDNA.

The purified cDNA was mixed with the supplied buffer (BRL) composed of 0.1M potassium cacodylate (pH 7.2); 1mM $CoCl_2$; 0.1M DTT and 15U terminal deoxynucleotidyl transferase (BRL). The reaction proceeded for 15 minutes at 37°C. The C-tailed product was recovered by the Geneclean™ procedure as described above (2.2.3).

2.2.5 Annealing of C-tailed cDNA to G-tailed *PstI* digested pBR322.

This procedure is a modification of that described by Huismans and Cloete (1987). The purified C-tailed cDNA was mixed with approximately 125ng of G-tailed *PstI* digested pBR322 cloning vector in the presence of 15mM NaCl; 0.2mM EDTA and 1mM Tris (pH 8.0). The annealing was carried out for 5 minutes at 80°C; 1 hour at 65°C; 1 hour at 56°C; 1 hour at 42°C and finally 1 hour at room temperature in a Hybaid™ thermocycler. The resulting mixture was then cooled on ice and transformed into competent *E. coli* HB101 cells.

2.2.6 Preparation of competent *E. coli* HB101 cells.

Cells were prepared for transformation by the standard $CaCl_2$ method (Sambrook *et al.*, 1989). Briefly, 1ml of an overnight culture of *E. coli* HB101 cells was inoculated into 60ml of LB (Luria Bertani) broth and grown at 37°C with shaking until an O.D of approximately $0.5_{(550nm)}$ was reached. The cells were spun down in a cooled Beckman J2-21 swing out rotor for 3 minutes at 3000 rpm and gently resuspended in 10ml fresh, ice cold 50mM $CaCl_2$. The

cells were then re-pelleted as described above, and resuspended in 1ml ice cold $CaCl_2$. The cells were left on ice for at least 60 minutes before being transformed.

2.2.7 Transformation of competent *E. coli* HB101 cells.

Half the DNA mix (15μl) was added to 150μl of the cells and then left on ice for 45 minutes. The cells were heat shocked at 42°C for 90 seconds (Maniatis *et al.*, 1989). This was then cooled on ice for 2 minutes. 850μl of prewarmed LB broth was added and the cells were incubated for 60 minutes at 37°C with shaking. The cell mixture was then plated out onto LB agar plates containing tetracycline (12.5μg/ml) and incubated overnight at 37°C.

2.2.8 Replica plating.

Colonies growing on tetracycline containing agar plates were replica plated onto LB agar plates containing ampicillin (0.1mg/ml). Colonies were lifted with sterile toothpicks and touched onto the ampicillin containing plates. The plates were then incubated at 37°C overnight.

2.2.9 Plasmid extraction.

Colonies were inoculated into 3ml LB broth supplemented with the appropriate antibiotic, and grown overnight at 37°C in a shaking incubator. Small scale alkaline lysis plasmid extractions were done on 3ml of culture (Sambrook *et al.*, 1989).

2.2.10 Dot Blot hybridizations.

In short, 0.5 - 1μg of plasmid DNA, which was denatured by being mixed with 100μl of a solution containing 0.4M NaOH; 25mM EDTA, was blotted onto a nylon N^+ membrane (Amersham). The probe was added to the membrane which was pre-hybridised at 42°C in a pre-hybridization solution composed of 50% deionized formamide; 0.5M NaH_2PO_4; 5mM EDTA (pH 7.4); 0.1% fat-free milk powder and 0.2% SDS for 40 minutes. The probe was prepared by *PstI* digestion of pBR322 containing a partial AHSV-9 segment 3 gene cloned into the *PstI* site, and resolving this by agarose gel electrophoresis. The desired fragment was excised from the gel and purified by the Geneclean™ procedure. One to 2μg of the fragment was labelled with 10μCi [α-^{32}P] dCTP by nick translation as described by the supplier (Promega).

After removal of the probe, the membrane was rinsed in 2xSSC (0.3M NaCl; 30mM trisodium citrate; pH7.0), followed by two washes for 10 minutes each in 2xSSC; 0.1% SDS. The first wash was done at 37°C and the second, at 65°C. The membrane was then autoradiographed overnight at -70°C.

2.2.11 Restriction enzyme analysis of recombinant plasmids.

A half to 1.0μg of the plasmid was digested with 5-10U of the relevant restriction endonuclease (Boehringer Mannheim) according to the supplier's specifications. The digested plasmids were analyzed by 1% agarose gel electrophoresis. Molecular weight markers were run in parallel to estimate the sizes of the resulting bands.

2.2.12 Sequencing.

For all sequencing reactions, the Sequenase™ 2.0 (USB) kit was used. Recombinant plasmids were purified by either CsCl density centrifugation or by a matrix adsorption kit (Nucleobond™). 2-3μg of plasmid DNA was denatured according to the supplier's specifications, and used directly in the sequencing reactions.

Primers used for sequencing were the pBR322 universal, forward and reverse primers, pBR322-*PstI*(+) [5' GCTAG AGTAA GTAGT T 3'] and pBR322 *PstI*(-) [AACGA CGAGC GTGAC 3']. Additional primers designed to recognise the 5' (SI5: 5' GGAGA TCTAT GCAAG GGAAT GAAAG AATAC 3') and 3' (SI3: 5' GGAGA TCTGG CTGCT AAATC GTTGG TCG 3') ends of the segment 3 gene, were also used.

2.3 Results.

2.3.1 cDNA synthesis.

A characteristic feature of the dsRNA genome segments of the Reoviridae, is the absence of poli-A tails at their 3' ends. The strategy developed by Cashdollar *et al.* (1982) for the cloning of this dsRNA was therefore to provide the 3' end with poli-A tails using the enzyme poli A polymerase. This provides a template which will allow cDNA synthesis to be primed by oligo dT primers.

The approach followed for the polyadenylation has been to denature the dsRNA prior to polyadenylation (Cashdollar *et al.*, 1982; Huismans and Cloete, 1987). However, the enzyme has been shown to be able to utilise non-denatured dsRNA as a template (Nuss and Dall, 1990). It is not known which approach yields better cDNA. A potential problem of using a denatured template as the substrate for the enzyme, is that any nicks present in the dsRNA would be exposed upon denaturation and will consequently be tailed. This will not occur in the case of non-denatured dsRNA, allowing their 3' ends to be more efficiently tailed, thereby potentially increasing the proportion of full length cDNA. To investigate which of the two approaches yielded better cDNA, the polyadenylated dsRNA was prepared by either polyadenylating a denatured dsRNA template, or a dsRNA template which was not denatured as described under 2.2.1. The AHSV dsRNA used in the polyadenylation was previously enriched in the medium sized genome segments by sucrose gradient sedimentation. cDNA synthesis on the two differently polyadenylated dsRNA templates was carried out as described in 2.2.2 and revealed that the non-denatured dsRNA template yielded higher counts in terms of [α-^{32}P]dCTP incorporation. The quality of the cDNA synthesised was then directly assessed by 2% vertical alkaline agarose gel electrophoresis (see fig 2.2). The cDNA derived from the non-denatured dsRNA (lane 1) revealed discrete bands representing the different segments of the AHSV genome, including the larger segments. In the sample

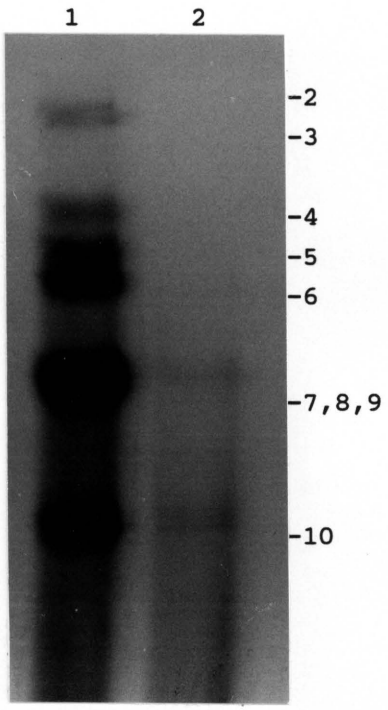

fig 2.2 Autoradiograph of AHSV-9 cDNA separated by 2% vertical agarose gel electrophoresis. Lane 1 is cDNA synthesized off a non-denatured dsRNA template. Lane 2 is cDNA synthesized from dsRNA which was denatured prior to polyadenylation. The different segments are indicated on the right hand side of the figure.

where the dsRNA had been denatured prior to polyadenylation (lane 2), discrete bands were only evident for the smaller genome segments. Also evident, although not shown in the figure, was a smear near the base of the gel in lane 2, which was largely absent in lane 1. This result suggests that much of the cDNA synthesised off the denatured poli-A dsRNA was small, and of an indiscrete size, therefore not representing full length genome segments. Based on these results with this sample of dsRNA used, non-denatured dsRNA yielded cDNA of much better quality in terms of the amount of full length segments synthesised, particularly for the larger genome segments. Consequently, the dsRNA was not denatured in subsequent cDNA reactions.

Of the cDNA synthesised, segment 3 only represents a small percentage of the total (fig. 2.2, lane 1). It was therefore necessary in subsequent steps to enrich the cDNA in segment 3 cDNA by size fractionation. This should result in an increased proportion of full length segment 3 clones.

2.3.2 Size fractionation and cloning of the Segment 3 cDNA.

i) Size fractionation.

There are two reasons why the cDNA is size fractionated. Firstly, to increase the proportion of segment 3 to be cloned, and therefore the efficiency of cloning that particular segment. The second reason why size fractionation is important, is to remove smaller incomplete segment 3 fragments of cDNA which may compete for the full length segment's single stranded cDNA during the annealing step.

For the size fractionation, two approaches were followed. After the alkaline hydrolysis of a scaled up cDNA reaction, the cDNA was either fractionated by alkaline sucrose gradient sedimentation, or by alkaline agarose electrophoresis. The best resolution of the different

cDNA fragments should be obtained by the electrophoresis method as the desired band can be excised directly. The sedimentation method should provide a mixture of different fragments of a similar size as the bands are too close together to be resolved completely. Both approaches were followed for the cloning of segment 3 to investigate which yielded a greater proportion of full length clones.

The medium sized pool of AHSV-9 dsRNA, which was used for the cDNA synthesis described in the preceding section (2.3.1), was found to contain a significant proportion of segment 3 cDNA following cDNA synthesis (fig 2.2 lane 1). This reaction was scaled up 5 times and fractionated by 10-40% (w/v) alkaline sucrose gradient sedimentation. Samples of fractions 11 to 21, encompassing the gradient peak, as determined by scintillation counting, were analyzed on a vertical 2% alkaline agarose gel. Fractions 11 to 13 (containing the largest cDNA) were pooled. Samples from the different fractions were run in parallel with an unfractionated cDNA control on a 2% vertical alkaline agarose gel (fig 2.3). Fractions 11 to 14 (lanes 2 and 3) appeared to contain mainly segment 3 cDNA. Some segment 4 cDNA is also present in fraction 14 (lane 3). The fractions containing the segment 3 cDNA were pooled and purified by Geneclean™.

For the 2% alkaline agarose gel fractionation, a total pool of sucrose gradient purified AHSV-3 dsRNA was used. Denaturation of this dsRNA prior to cDNA synthesis was omitted based on results obtained from the AHSV-9 cDNA synthesis. The relevant segment 3 cDNA was excised after autoradiography of the wet gel. The purified fragment was then extracted from the agarose by the Geneclean™ procedure.

The two methods of size fractionation were judged on the basis of the clones resulting from the different approaches. Since the cDNA was derived from two different pools, the comparison between the two methods will be qualitative, i.e. in terms of proportions of full length segment 3 clones and not absolute numbers of clones.

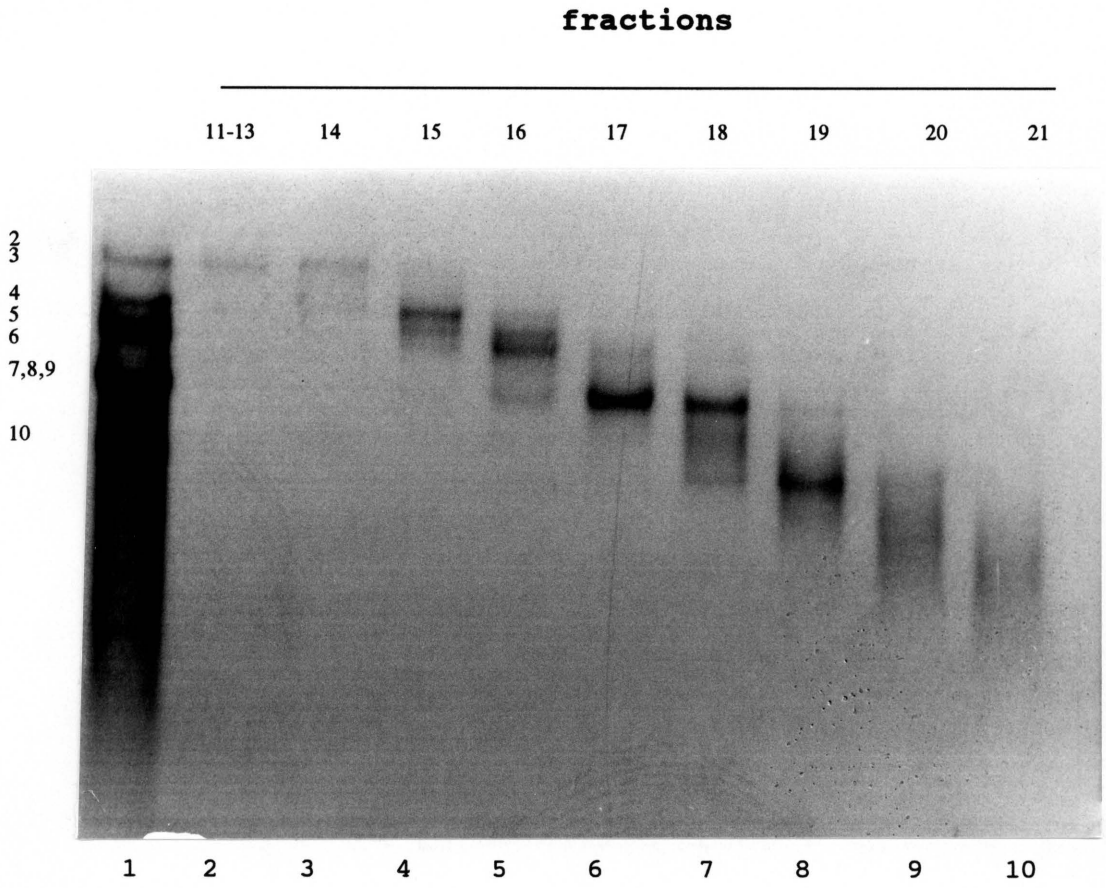

fig. 2.3 Autoradiograph of a 2% vertical agarose gel of cDNA fractionated by alkaline sucrose gradient sedimentation. Lane 1 is an unfractionated cDNA control. Lanes 2-10 are the various cDNA fractions. The genome segment positions are indicated on the left hand side of the figure.

ii) Analysis of clones.

To evaluate the different fractionation strategies, the clones needed to be analyzed to see what proportion of the two approaches yielded full length segment 3 clones.

Since the genes were cloned into the ampicillin resistance gene of pBR322, an ampicillin sensitive phenotype was indicative of the presence of a cloned insert. Recombinants resulting from the transformation could therefore be identified by replicating colonies onto LB agar plates supplemented with ampicillin after initial selection of transformants on tetracycline plates. To identify clones with a segment 3 insert from the two serotypes, the ampicillin sensitive colonies were screened for the presence of the segment 3 gene by Dot Blot hybridization using a partial AHSV-9 segment 3 clone, containing 1.2Kb of the 5' end of the gene, as a probe (provided by B. Greyling, dept Genetics, U.P). This probe should be suitable for the detection of AHSV-3 segment 3 DNA because of the conserved nature of this gene (Bremer *et al.*, 1990).

The results of the Dot Blot for the AHSV-9 cloning yielded ten segment 3 specific recombinants. This equates to approximately 10% of the total number of clones obtained from the cloning experiment. In the case of AHSV-3, only one segment 3 specific clone from a total number of 10 clones was obtained. To assess the size of the segment 3 inserts cloned into the *PstI* site of pBR322, *PstI* digests were carried out. The results of the *PstI* digests for the AHSV-9 segment 3 clones (fig 2.4) revealed some size variation ranging from between approximately 1.5Kb (lane 8) to 2.8Kb (lane 1), as determined from their relative migration with respect to the size marker (lane 2) which was run in parallel. The 2.8Kb insert (lane 1), which was designated I74, corresponds to the full length size of segment 3 published for AHSV-4 (Iwata *et al.*, 1992). The single AHSV-3 segment 3 clone was also deemed full length on the basis of a *PstI* digest (result not shown), and was designated D9. The rest of the clones obtained from the AHSV-3 cloning experiment were also digested by

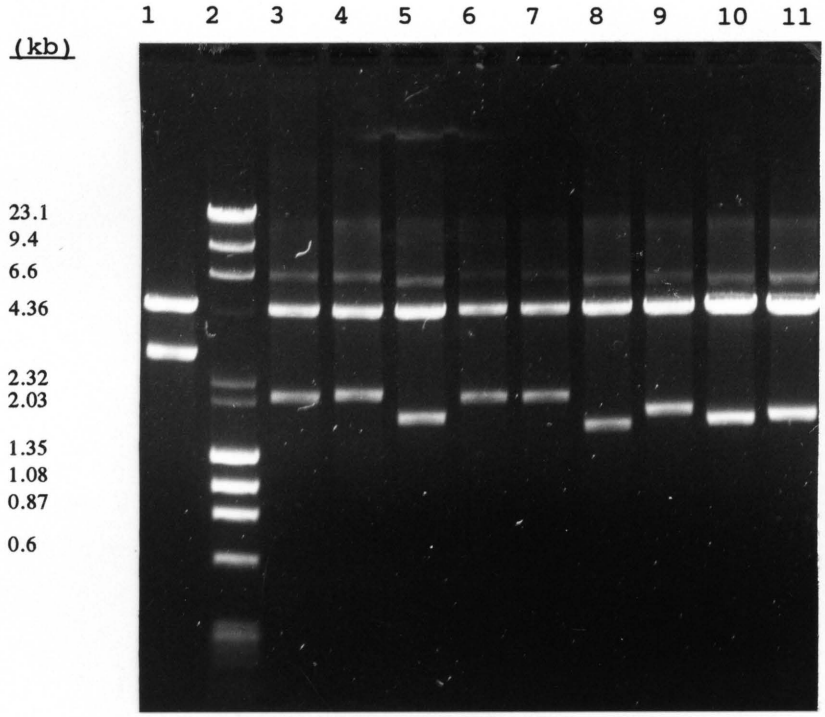

fig 2.4 Ethidium bromide stained 1% agarose gel electrophoretogram of the *PstI* digested AHSV-9 cDNA cloned into the *PstI* site of pBR322. Lane 1 represents the full length recombinant, I74. Lanes 3-11 are the incomplete sized AHSV-9 segment 3 clones. Lane 2 is molecular weight marker II and *HaeIII* digested ϕX 174 DNA (Boehringer Mannheim). The sizes are indicated on the figure, in kilobases (Kb).

PstI, and the size variation obtained ranged from 0.3Kb to 2.8Kb (the segment 3 clone) although these clones were not assigned. A total of three of these clones were larger than 2Kb.

To more accurately assess whether the segment 3 recombinants of AHSV-3 and AHSV-9 (I74 and D9 respectively) were full length, the termini of the constructs were sequenced to see if the ends corresponded to the termini of the published AHSV-4 segment 3 sequence (Iwata *et al.*, 1992).

2.3.3 Sequence analysis of the termini of the cloned segment 3 genes of AHSV-3 and AHSV-9.

Based on the *PstI* digests of the cloned segment 3 genes of AHSV-3 and AHSV-9, it seemed evident that they were very close to being full length. To confirm this, the termini of the constructs were sequenced. Due to the high sequence conservation of the segment 3 gene (Iwata *et al.*, 1992) the ends should be easily identifiable. In addition to this, sequencing of the termini should allow the estimation of the level of conservation of the segment 3 gene within the AHSV serogroup.

Sequencing was carried out using pBR322 universal forward and reverse primers, pBR322-*PstI*(+) and pBR322-*PstI*(-). Due to the presence of GC tails, introduced as an artefact of the cloning procedure, only limited sequence data could be obtained using these primers. Consequently internal primers (SI5 and SI3) were constructed based on sequence information obtained with the pBR322 universal primers (which could also be subsequently used to amplify the open reading frame of the gene which was required for a subsequent application).

From comparisons with the AHSV-4 sequence, the following could be ascertained in terms of the non-coding bases. The AHSV-9 clone (I74) (fig 2.5) lacks 4 non-coding bases at the 5' end (fig 2.5a) leaving 22 preceding the initiation codon. At the 3' end, 39 base pairs were missing (fig 2.5b), with 9 remaining non-coding bases after the stop codon. In the case of the AHSV-3 recombinant, the 5' end (fig.2.5a) was 24 bases short of being full length and only two of the non-coding bases were present. The gene did however, possess the entire 3' end with the identical 48 non-coding bases present in the AHSV-4 VP3 gene sequence (fig.2.5b).

The bases that were present in the non-coding regions of both the 5' and 3' termini of these segment 3 genes show an identity of 100%.

Based on the sequencing, it could be concluded that both the AHSV-3 and AHSV-9 segment 3 clones were full length with respect to their open reading frames, but not so with respect to the non-coding bases.

In order to assess whether the segment 3 gene of AHSV displays a similar level of conservation as is found amongst the BTV serogroup, sequence comparisons of the open reading frame termini were done. Of the 5' end, 191 bp of AHSV-3 and AHSV-9 were sequenced, and 239 bp from the 3' end. This represents 7% of the total gene sequenced from the 5' end and 9% of the total from the 3' end, which comprises 16% of the total gene length. The sequencing results are shown (see fig 2.6) alongside the published AHSV-4 sequence (Iwata *et al.*, 1992). The results are summarised in table 2.1, where the average homology over the segment 3's of these three serotypes over the region sequenced, is 95.5%. 82% of this change is at the third base of the codon, and therefore predominantly silent.

On the basis of these results, the segment 3 genes of a sample of the AHSV serogroup appears to be highly conserved.

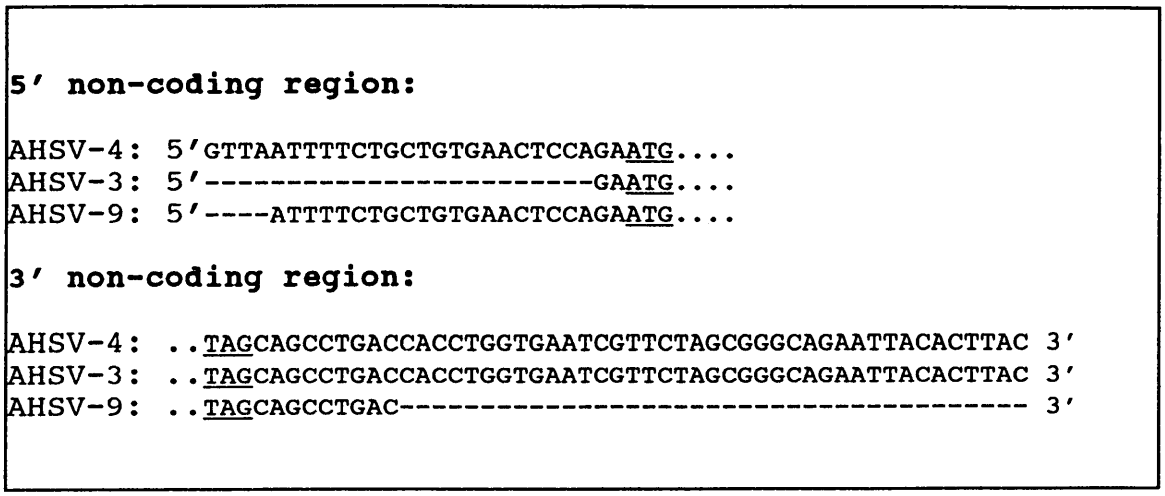

fig 2.5 **Comparison of the non-coding sequences of the segment 3 genes of AHSV-3 , AHSV-9 and AHSV-4.**

Underlined bases represent the start (fig 2.5a) and stop (fig 2.5b) codons, and the dashed lines represent bases missing from the segment 3 clones.

A)

```
AHSV-4: 5'  ATG.CAA.GGG.AAT.GAG.AGA.ATA.CAA.GAT.AAA.AAT.GAG.AAG.GAG.AAG.GCG
AHSV-3: 5'                      A                                 G  G       T
AHSV-9: 5'                      A                                 A  C       C

AHSV-4:     .TAT.GCA.CCA.TAT.CTT.GAC.GGA.GCG.AGC.GTA.TCA.ACG.GAT.AAT.GGG.CC
AHSV-3:              A       T                                          G
AHSV-9:              G       C                                          A

AHSV-4:     C.ATT.TTA.TCA.GTG.TTT.GCC.TTA.CAG.GAA.ATC.ATG.CAA.AAA.ATT.AGG.C
AHSV-3:                  T                                      G
AHSV-9:                  C                                      G

AHSV-4:     AA.AAT.CAG.AGC.GAT.ATG.GCT.GCA.CAT.GCT.CCC.GAT.GTT.GAT.GGG.GCG.
AHSV-3:
AHSV-9:

AHSV-4:     ATA.CCA.GAG.GTA.ATG.ACT.ATA.ATC.AGT.GG 3'
AHSV-3:                        T       C
AHSV-9:                        G       T
```

B)

```
AHSV-4:     TG.GTC.GCT.TTT.AAA.TAC.GCG.ACA.ACC.GCG.ACT.GCC.TAT.TTC.TTA.TTG.
AHSV-3:                            A                   C
AHSV-9:                            A                   T

AHSV-4:     TAT.AAC.GTT.GAG.TAT.TCA.AAT.ACG.CCA.GAT.ACT.TTG.ATC.ACA.GTG.AAC
AHSV-3:                                                G           G
AHSV-9:                                                A           A

AHSV-4:     .CCA.ACA.TTT.ACG.ATG.ACG.AAA.ATT.CAT.ATG.CGA.AAG.AAA.ATC.GTT.AG
AHSV-3:       A                      G               G       A   T   C
AHSV-9:       C                      A               G       G   T   C

AHSV-4:     A.CGA.GTT.CGC.GCT.CCA.GAT.GTG.CTA.TCA.CAA.GTT.AAC.AAA.CGT.TTA.G
AHSV-3:             T                                G   T
AHSV-9:             C                                A   C

AHSV-4:     TC.GCG.TAC.AAA.GGT.AAG.ATG.CGC.TTA.ATG.GAT.GTA.ACG.AAA.TGC.CTA.
AHSV-3:      T   G   C          G       T
AHSV-9:      C   A   T          A       T

AHSV-4:     AAG.ACT.GGA.GTT.CAA.CTT.GCG.CGA.CCA.ACG.ATT.TAG. 3'
AHSV-3:           C             CC
AHSV-9:           T             TC
```

fig 2.6 Comparison of the termini of the coding regions of the segment 3 genes of AHSV-3, AHSV-9 and AHSV-4 (Iwata *et al.*, 1992). **A)** 5'end coding regions of the segment 3 genes of AHSV-3, AHSV-4 and AHSV-9. When one notes the highlighted bases of the AHSV-4 sequence, it appears that an error may have been made in the recording of the sequence. This has been concluded since both AHSV-3 and AHSV-9 exhibit a different sequence. In addition to this, the error stretches over two codons. Due to the apparent conservation of the gene, it seems unlikely that such a change would have occurred. The published triplet is AGA. **B)** Comparison of the 3'end coding regions of the segment 3 genes of AHSV 3, 4 and 9. Full stops indicate codon intervals and underlined bases, non silent amino acid changes.

Table 2.1: Nucleic acid comparisons between the segment 3 genes of AHSV-3, AHSV-4 and AHSV-9.

Nucleic acid comparisons.	AHSV-4	AHSV-9
AHSV-3		
% identity	97.1%	94.9%
AHSV-4		
% identity		94.5%

2.4 Discussion.

Segment 3 cDNA copies of AHSV-3 and AHSV-9, which were full length in terms of their open reading frames, were cloned. The size of both the segment 3 clones conformed to the anticipated size of 2.8Kb (Iwata *et al.*, 1992) similar to that of the segment 3 genes of BTV and EHDV (Ghiasi *et al.*, 1985; Wilson, 1991).

The genes were cloned essentially by the technique pioneered by Cashdollar *et al.* (1982) which entailed polyadenylating the dsRNA genome and then synthesising cDNA by priming with oligo dT primers. This technique has proved successful in the cloning of many orbivirus genes, including the segment 3 gene of BTV (Purdy *et al.*, 1984) and the segment 3 gene of AHSV-4 (Iwata *et al.*, 1992). However, this AHSV segment 3 gene could not be successfully expressed in the baculovirus system for as of yet, unknown reasons (Huismans, pers. comm). It was therefore of interest to clone segment 3 genes of other serotypes to investigate whether they could be expressed.

The cloning of orbivirus dsRNA has previously involved the poli-A tailing of previously denatured dsRNA (Cashdollar *et al.*, 1982; Huismans and Cloete, 1987). However, it has been shown that dsRNA in its native form provides a suitable template for poly A polymerase (Nuss and Dall, 1990). In our hands, good quality cDNA consisting of a high proportion of full length cDNA segments was only achieved when the dsRNA was not denatured prior to polyadenylation. The reasons for this were more fully investigated by Huismans and Napier (manuscript in preparation), but probably involves the fact that this approach eliminates the tailing of nicks within the RNA made available by the denaturation. These internally tailed substrates would compromise the efficiency with which the true 3' ends of the genes would be tailed. This would cause much of the cDNA to be synthesized off these shortened templates.

cDNA which is not full length may compete for the full length complementary templates during the re-annealing step of the cDNA synthesis. Consequently, the cDNA is size fractionated prior to this annealing step. Two approaches were followed for this size fractionation. The cDNA was either size fractionated by alkaline sucrose gradient sedimentation as described by Huismans and Cloete (1987) or alkaline agarose gel electrophoresis (Huismans *et al.*, 1987d). The former approach has proved successful for the cloning of large BTV genome segments, however, large genome segments could not be cloned by the latter approach (Huismans *et al.*, 1987d). In this study both approaches proved successful for cloning of full length segment 3 genes of AHSV, however, direct comparisons could not be drawn, as different pools of RNA were used for the cloning experiments. A feature of the gel fractionation approach, was the presence of clones containing very small inserts (0.3Kb). This may have been as a result of the gel being overloaded. The gradient approach contained less size variation (ranged between 1.5Kb to 2.8Kb for the segment 3 clones).

A large body of evidence based on immuno-cytochemical (Inumaru *et al.*, 1987); hybridization (Huismans and Cloete, 1987; Bremer *et al.*, 1990)) and gene sequencing (Iwata *et al.*, 1992; Hwang *et al.*, 1994; Wilson, 1991; Gould and Pritchard, 1991) has shown that VP3 is a conserved protein in the orbiviruses. It has been found to be the most conserved of the major structural genes (Iwata *et al.*, 1992). This conservation is reflected when viruses are compared between serotypes within a serogroup, where for example, between 4 U.S. BTV serotypes (10, 11, 13 and 17) the nucleic acid homology is 97% (Hwang *et al.*, 1994). A similar situation prevails in the EHDV serogroup, where the amino acid identity between serotypes 1 and 2 is 95% (Wilson, 1991). Based on these results, a similar level of conservation could be expected for the AHSV serogroup.

Approximately 16% of the AHSV-3 and AHSV-9 segment 3 genes were sequenced at the open reading frame termini. Of the total sequenced, 7% of the sequence was obtained from

the 5' end and 9% from the 3' end. These results revealed 95% homology between these two serotypes. When compared with the published AHSV-4 sequence (Iwata *et al.*, 1992), over the same region, the homology between AHSV-4 and AHSV-9 is 95%, and between AHSV-3 and AHSV-4, it is 97%.

It was found that the conservation over the regions sequenced for the AHSV-3 and AHSV-9 genes is an accurate representation of the conservation over the entire gene. When the segment 3 genes of three different BTV serotypes (10, 11 and 17) are compared, the conservation over the entire gene is 97.7%, and over the regions corresponding to the termini sequenced for the AHSV-3, and AHSV-9, it is 96.3%. When similar comparisons are done between the segment 3 genes of AHSV-4 and the segment 3 gene of BTV-10, the conservation over the entire gene is 59.7%, and over the termini, it is 58%. Based on these observations, the conservation at the termini, i.e over the region sequenced for the AHSV-3 and AHSV-9, reflects the conservation over the entire gene. These results suggested that sequence data covering the entire segment 3 genes of AHSV-3 and AHSV-9 would provide little additional information over the genetic conservation of these genes.

It can therefore be concluded that the conservation between AHSV serotypes for the segment 3 gene follows a similar trend to the conservation seen in other orbivirus serogroups, such as BTV and EHDV. This is at least true for the AHSV-3, AHSV-9 and AHSV-4 serotypes.

Chapter 3. EXPRESSION OF THE AHSV VP3 GENE PRODUCT.

3.1 Introduction.

The development of the baculovirus expression system has enabled many studies that are directed towards the structure-function analysis of viral proteins. This has lead to a much greater understanding of the orbivirus structure (reviewed in Roy, 1992; 1996).

The baculovirus expression system has the capacity to express foreign proteins in large amounts when expressed under the control of the polyhedrin promoter. The polyhedrin gene is non-essential in tissue culture (Miller, 1988; O'Reilly *et al.*, 1992). The levels of protein synthesized from this promoter (switched on late in the infection cycle) can be as high as 20 - 50% of the total cellular protein, depending on the gene being expressed (O'Reilly *et al.*, 1992). The baculovirus expression system has the additional advantage that the proteins expressed in the insect cells will undergo most of the post-transcriptional and post-translational modifications associated with the proteins. Baculoviruses have a narrow insect host range, and are unable to replicate in mammalian cells. This feature makes them relatively safe in terms of biological containment (O'Reilly *et al.*, 1992).

Much progress has been made in defining the structure of the major inner capsid proteins of orbiviruses and the way they are assembled into subviral structures. This progress has been greatly facilitated by the baculovirus expression system. The ability to selectively express certain genes in combinations has made it possible to define the minimum components required for some of the assembly processes involved in orbivirus morphogenesis. When the major core proteins, VP3 and VP7 of BTV are co-expressed in insect cells, they are able to spontaneously assemble into structures resembling the core of the virus in the absence of any other viral proteins (French and Roy, 1990). By using this system it was also shown that the formation of these structures is dependent on the presence of both of these proteins, as

the expression of a VP7 containing a specific point mutation in the presence of a native VP3 abrogates the assembly of these core-like particles (Le Blois and Roy, 1993).

Subviral particle expression has allowed the elucidation of functional differences between other orbiviruses and for other members of the Reoviridae family. Studies with Broadhaven virus (a tick-borne orbivirus), have shown that the VP7 component is not required for the stable assembly of subcores. The VP3 analogue (VP2), expressed alone is capable of spontaneously assembling into these structures (Moss and Nuttall, 1994). The VP3 analogue in rotavirus (also VP2), also spontaneously assembles into core-like particles without the aid of additional rotavirus proteins (Labbé *et al.*, 1991). Since the VP3 gene of AHSV is closely related to its cognate gene in BTV (Pritchard *et al.*, 1995), one may expect that both VP3 and VP7 will be required for its assembly into a core-like particle structure.

The assembly of core-like particles in insect cells has proved to be a useful functional assay in identifying the specific amino acids of the major core proteins involved in this process (Le Blois *et al.*, 1991; Le Blois and Roy, 1993; Tanaka and Roy, 1994; Tanaka *et al.*, 1995). This was done indirectly by showing that the VP3 protein of EHDV could be substituted with its BTV counterpart, and still assemble into authentic core-like particles (Le Blois *et al.*, 1991), and then more directly, by deleting and substituting amino acids in the BTV VP3 and investigating their assembly into these structures (Tanaka and Roy, 1994; Tanaka *et al.*, 1995).

Questions about how the minor structural proteins interact with the core particle are now also beginning to be addressed by co-expressing these proteins together with VP3 and VP7 and then studying the resulting structures (Loudon and Roy, 1991). In these studies they were able to show that the minor structural protein VP1 of BTV is incorporated into the core-like particle and interacts directly with the VP3 component of the particle.

Until now the VP3 gene of AHSV has not been expressed as a baculovirus recombinant. It has been cloned as a full length gene (Iwata *et al.*, 1994), but attempts to express it have so far, proven unsuccessful (Huismans pers. comm). This suggested the need to test the expression of the VP3 genes of different serotypes, in alternative expression systems such as in mammalian cell lysates or in a bacterial expression system, to investigate whether this was specifically a problem of the baculovirus system being unsuitable for AHSV VP3 gene expression. This does however seem unlikely as the cognate BTV gene has been successfully expressed as a baculovirus recombinant (Inumaru *et al.*, 1987).

Besides the potential inherent problems associated with AHSV VP3 expression, the method in which the VP3 cDNA is tailored for expression may present an additional problem. The GC tails, which are an artefact of the cDNA synthesis strategy, are removed by a PCR based strategy (Nel and Huismans, 1991). Due to the error prone PCR, where incorporation of incorrect nucleotides is a problem, the introduction of nonsense mutations may occur. This presents another reason for expressing AHSV VP3 in alternative systems i.e, to see whether the correct sized products are synthesised, or if perhaps nonsense errors had been introduced.

3.2 Material and methods.

3.2.1 Cells and virus.

AHSV used for protein analysis were obtained from the OVI. The virus was propagated by limited passage in baby hamster kidney cell (BHK-21) or CER cells, grown as monolayers in Roux flasks or roller bottles, as described by Huismans (1979) using modified Eagle's medium supplemented with 5% bovine serum albumen (Verwoed, 1969). The *lacZ* recombinant *Autographica calafornica* nuclear polyhedrosis virus (AcRP23-*lacZ*) (Possee and Howard, 1987) and *Spodoptera frugiperda* insect cells were obtained from the NERC Institute of Virology, Mansfield road, Oxford, UK. Cell culturing and handling technique were done essentially as described by Summers and Smith (1987).

3.2.2 PCR amplification of the segment 3 cDNA clones.

Two primers were designed to amplify the segment 3 cDNA clones of AHSV-3 and AHSV-9, which were cloned into the *PstI* site of pBR322 (section 2.3.2). The forward primer, [2]SI5 (5' GG*AGA TCT*AT GCAAG GGAAT GAAAG AATAC 3'), was designed to include, at its most 5' end, the gene's start codon, in it's amplification. The reverse primer, SI3 (5'GG*AGA TCT*GG CTGCT AAATC GTTGG TCG 3') was designed to include the stop codon in its amplification, and arbitrarily, 5 non-coding bases. For the PCR reaction, the following components were mixed together in a 0.5ml tube: 50ng of the I74 or D9 template; 100 pmoles of the SI5 and SI3 (whose fidelity was verified by their ability to sequence their derivative gene); 1.5mM $MgCl_2$; between 50-200μM each dNTP; buffer (50mM KCl; 10mM Tris-HCl [pH 9.0]; 0.1% Triton X-100) and 2.5U of Taq DNA polymerase (Promega). The

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[2] Italicised bases represent the BglII restriction sites, and the underlined bases, in SI5 represent segment 3 start codon, while the underlined bases in SI3 represent the stop codon on the complementary strand.

thermo-cycling was carried out in a Hybaid™ thermocycler with the following cycling conditions: 30 cycles at 95°C/5min; 51°C/45s; 72°C/3min. During the first cycle, the denaturation step (95°C) was continued for 5 minutes, and during the last cycle, the elongation step was continued for 5 minutes at 72°C. After the reactions were completed, 1/10 of the product was analyzed by 1% agarose gel electrophoresis.

3.2.3 C-tailing and cloning of PCR products.

The PCR product from a single reaction (approximately 2μg) was electrophoresed in a 0.8% agarose gel. The products were excised from the gel to get rid of primer dimers. The DNA was purified from the gel slice using Geneclean™ of which 0.6 to 1μg was C-tailed (2.2.4). The C-tailed product was recovered using Geneclean™ as described in 2.2.3. The dCTP tailed PCR products were annealed to G-tailed *PstI* digested pBR322 (BRL) (2.2.5), and then transformed into competent HB101 *E. coli* cells (2.2.6 and 2.2.7).

3.2.4 Colony blot hybridization.

Colonies resulting from the transformation were lifted onto a Hybond-N membrane (Amersham). The membrane was then soaked for 7 minutes in a solution containing 0.5M NaOH; 1.5M NaCl, and then twice for 3 minutes in a second solution composed of 1mM EDTA; 1.5M NaCl; 0.5M Tris (pH 7.4). The membrane was finally soaked in 2xSSC to equilibrate. The membrane was then sealed off in a plastic bag, and fixed for 5 minutes per side, by ultra-violet exposure. The membrane was then pre-hybridized (2.2.10) for 40 minutes at 42°C.

The *PstI* fragment of I74 encompassing the whole open reading frame, was used as a probe. This was excised from a 1% agarose gel, and purified by Geneclean™. Approximately 1μg

was labelled by nick translation (see 2.2.10) and then added to the membrane as described (2.2.10).

3.2.5 Partial restriction enzyme digestion.

A volume of 10μl (5.0μg) cesium chloride purified AHSV-3 PCR product cloned into *PstI* site of pBR322 was added to 6μl of 10x concentration buffer for restriction enzyme digestion (Boehringer Mannheim), consisting of 100mM Tris.HCl (pH7.$5_{37 \cdot C}$); 100mM $MgCl_2$; 0.5M NaCl; 10mM DTT. The volume was adjusted to 60μl with UHQ water and aliquoted into 9 eppendorff tubes with 6μl in each, except for the first tube, which had 12μl of the mixture. 5U of *BglII* was added to the first tube. This was mixed well and half the volume was removed from the tube and added to the second tube. This process was repeated through to the ninth tube from which 6μl was discarded. The digestion mixtures were incubated at 37°C for 60 minutes, and then loaded onto a 0.8% agarose gel for subsequent analysis in the presence of DNA molecular weight markers.

3.2.6 *In vitro* transcription.

The AHSV-3 segment 3 PCR product was cloned into the *BamHI* site of the transcription vector, Bluescribe (pBS) (Stratagene). The orientation was determined by the presence of a *HindIII* site 0.7Kb from the 3' end of the gene, and another *HindIII* site in the multiple cloning (MCS) site of pBS. The recombinant plasmid was linearised at a unique *XbaI* site downstream of the 3' end of the cloned insert in the pBS MCS. The linearised plasmid was purified by phenol/chloroform extractions and ethanol precipitated. The precipitated plasmid was resuspended in DEPC treated UHQ water.

The *in vitro* transcription reactions were carried out according to the supplier's instructions (Promega) which was as follows: 1.4μg of linearized template DNA was added together with

40U of T7 RNA polymerase; transcription buffer containing a final concentration of 40mM Tris-HCl (pH8.0); 6mM $MgCl_2$; 10mM DTT; 2mM spermidine; 30U RNase inhibitor and 0.187mM to 0.75mM of each rNTP (ribo-nucleotide triphosphate). The final reaction volume was made up to 20μl with DEPC treated UHQ water. This mix was incubated at 37°C for 60 minutes. The product was then analyzed on a 1% agarose gel, which was prepared in freshly autoclaved, sterile 1xTAE buffer.

3.2.7 *In vitro* translation.

The *in vitro* translation reaction was carried out in a cell free rabbit reticulocyte lysate™ (Amersham). Approximately 3μg (6μl) of mRNA was added to 16μl of lysate and 10μCi S^{35}-methionine. The reaction was incubated at 30°C for 60 minutes. A 5μl sample of this was denatured by adding an equal volume of 2x protein solvent buffer (PSB) (125mM Tris.HCl (pH $6.8_{r.t}$); 4% SDS; 20% glycerol; 10% β-mercapto-ethanol; 0.002% bromophenol blue). This was vortexed for 1 minute, boiled for a further 3 minutes and then centrifuged for 1 minute in a benchtop centrifuge. The supernatant was subsequently loaded onto a 12% discontinuous SDS polyacrylamide gel as described by Laemmli (1970) and electrophoresed at 120V. The gel was then fixed and stained in a solution composed of 0.125% Coommasie Blue; 50% methanol and 10% acetic acid and then counter-stained in 5% glacial acetic acid and 5% ethanol. This was followed by signal amplification using AMPLIFY™ (Amersham) for 30 minutes. The gel was dried and autoradiographed for 48 hours at -70°C.

3.2.8 Immune precipitation.

For the immune precipitation, 10μl of the *in vitro* translated product was mixed with 5μl of concentrated αAHSV-4 rabbit serum and diluted to 60μl with 0.1M STE buffer (0.1M Tris (pH 7.4); 0.01M EDTA; 0.1M NaCl). This was shaken at room temperature on an orbital shaker for 60 minutes. To this, 30μl of *Staphylococcus aureus* slurry (10% w/v), washed

and resuspended in 0.1M STE buffer, was added and shaken for 60 minutes at room temperature. The mix was then spun for 30s in a benchtop centrifuge and the supernatant discarded. The pellet was resuspended in 100μl STE buffer and spun for 30s in a benchtop centrifuge. The washing step was repeated twice, and the pellet, which was resuspended in 20μl 2xPSB, was vortexed and boiled for 3 minutes. Insoluble material was removed by brief centrifugation. The supernatant was loaded onto a 12% discontinuous SDS polyacrylamide gel and autoradiographed as previously described (3.2.7).

3.2.9 *In vivo* radio-labelling of CER cells infected with AHSV.

AHSV-3 stock, in 6ml of Eagle's medium was added to a fresh monolayer of CER cells. Infections took place at 37°C for 90 minutes, at which time the supernatant was replaced with 10ml Eagle's medium lacking foetal calf serum (FCS), and incubated for 18 hours at 37°C. The cells were then rinsed once with methionine free Eagle's medium, and the volume replaced with 6ml of the same medium and left for 60 minutes at 37°C. Following this, 20μCi S^{35}-methionine was added and the incubation continued for 180'. The cells were harvested by detaching them from the surface using glass beads. The medium containing the detached cells was centrifuged for 5' at 3000 rpm in a J2-21 fixed angle rotor (Beckman) and washed twice in PBS. The cells were then resuspended in 0.1M STE buffer supplemented with 0.5% Triton X-100 for cell lysis. The nuclei were removed by low speed centrifugation.

3.2.10 Induction of GST bacterial fusion protein expression.

PCR products of the AHSV-9 segment 3 gene, were cloned directly into the *BamHI* site of the pGEX series of bacterial expression vectors (Smith and Johnson, 1988) by *BglII* terminal restriction of the PCR product, or cloned indirectly by the C-tailing approach into G-tailed pBR322 (see 3.2.3), and then subcloned by *BglII* restriction into the *BamHI* site of the pGEX

vectors. Recombinants in the correct orientation were grown overnight in LB broth supplemented with ampicillin and then diluted 10-fold by adding 1ml of cells to 9ml of ampicillin containing LB broth. The cultures were then incubated at 37°C by shaking for 120 minutes. The cultures were incubated for another 120 minutes in the presence of IPTG, which was first added to a final concentration of 0.5mM, and then after 60 minutes, a further 0.25mM IPTG was added. The cells were harvested by centrifugation of the cultures in a benchtop centrifuge for 90 seconds. The resulting supernatant was discarded, and the cell pellet washed once by being resuspended in 1ml phosphate buffered saline solution (PBS) and discarding the supernatant after centrifugation. The cells were finally resuspended in 50μl PBS. 5μl of the cell suspension were treated with an equal volume of 2xPSB for lysis and solubilization of the proteins. These were then vortexed for 1 minute and boiled for a further 4 minutes. The samples were sonicated for 5 minutes, followed by brief centrifugation before being loaded onto an 12% discontinuous SDS polyacrylamide gel for analysis. The gel was electrophoresed, fixed and stained as described in section 3.2.7.

3.2.11 Isolation of AcRP23 *lacZ* DNA.

$1.5x10^8$ *spodoptera frugiperda sf* 9 cells (at a density of 1.8×10^6 cells/ml) were infected with AcRP23 *lacZ* virus stocks (derived from a single blue plaque) at a multiplicity of 0.1 plaque forming units (PFU) per cell. This was done by first concentrating the cells by low speed centrifugation (3000 rpm for 5 minutes) and then adding the virus in a final volume of 20ml. The infection was done for 60 minutes at room temperature and then diluted back to the original volume of 80ml. The infection was continued for 4 days at 27°C in a spinner flask. Following this, the culture was centrifuged at 550g for 10 minutes in a SW28 rotor (Beckman), and the pellet, containing cellular material, discarded. The virus contained in the supernatant was pelleted by centrifugation for 90' at 9000g in a SW28 rotor. The resulting pellet was resuspended in 2ml 0.1x TE and homogenized by gentle syringing. This

was then layered onto a 5%/50% (w/v) sucrose cushion and spun for 90 minutes at 9000g in a SW28 rotor. The visible virus band was drawn off with a syringe, diluted 10-fold with 0.1 x TE and the virus, re-pelleted by centrifugation at 9000g in a SW28 rotor for 90 minutes. The resulting viral pellet was resuspended in 1ml 0.1x TE. To this, 200μl of 10% sarcosyl was added and this was incubated for 60 minutes at 60°C. This mixture was diluted to 3ml with 0.15M STE and supplemented with Tris buffer (pH 8.0) to a final concentration of 0.5M. An equal volume of a 1:1 phenol:chloroform mix was added, and the mix, gently swirled and centrifuged for 5 minutes at 4000 rpm in a J2-21 swing-out rotor (Beckman). The upper aqueous phase was collected and 1ml of 0.15M STE was added to the phenol phase and the extraction repeated. The aqueous phases were pooled and 2 volumes of 96% ethanol, added and the DNA precipitated at 4°C. The visible strands of DNA were either lifted directly with a pipette tip, or spun out from the ethanol by low speed centrifugation (5 minutes at 5000 rpm). The DNA was then air dried at room temperature, and resuspended in 0.1x TE. Samples of the viral DNA were analyzed by 0.5% low gelling temperature agarose gel electrophoresis at 50V.

3.2.12 Co-transfection of *sf9* cells with linearized AcRP23 *lacZ* DNA and recombinant baculovirus transfer vector.

A sample of AcRP23 *lacZ* DNA was digested with *Bsu 36i* at a unique *Bsu 36i* site for 240 minutes at 37°C. The extent of digestion was monitored on a 0.5% low melting agarose gel. The enzyme was then inactivated by heating for 15 minutes at 70°C. 1.3μg of the linearized DNA and 1μg of super-coiled, CsCl-purified recombinant baculovirus transfer vector were mixed together with 12.5μl of Lipofectin™ (BRL) and 5μl of UHQ water just prior to transfection, in a sterile polystyrene container. 1.8×10^6 *Spodoptera frugiperda sf9* cells were seeded in 6cm sterile tissue culture wells. The medium was removed from the cells and replaced with 2ml FCS free medium, in the presence of antibiotics. This was repeated once to ensure the removal of serum. The cells were covered with 1ml FCS free medium. To

this, the DNA-lipofectin mix was added. The cells and DNA were left overnight at 27°C. The FCS free Grace's insect medium was then replaced with Grace's insect medium with FCS and incubated for a further 4 days at 27°C. The supernatants were collected at 2, 3 and 4 days post infection and titrated to allow selection and purification of the recombinant virus.

3.2.13 Selection of potential baculovirus recombinants.

6 cm sterile tissue culture plates were seeded with $1.8x10^6$ *sf9* cells. A serial dilution (15 to $1.5x10^7$ times) of the virus containing supernatants obtained in 3.2.12 were diluted to 1ml with Grace's insect medium containing both antibiotics and FCS. The medium was removed from the seeded cells and replaced with the virus dilutions. Infections took place for 60 minutes at room temperature at which time the supernatants were removed and replaced with 50% Grace's medium (FCS^+ and $Antibiotic^+$) in 1.5% sterile, low melting agarose. The cells were then incubated for 4 days at 27°C. 1ml of a 0.1mg/ml solution of Neutral red (Sigma) containing 120μg/ml X-gal in Grace's medium, was added to the cells and incubated for 5 hours at 27°C, and then removed and left overnight at 27°C to allow visualization of the plaques.

3.2.14 Screening for baculovirus recombinants by dot blot hybridization.

Plaques were picked and placed in 1ml Grace medium (FCS^+ and $Antibiotic^+$), which was vortexed until the agarose plugs had disintegrated. Sterile tissue culture wells (16mm) were seeded with $1.4x10^5$ *sf9* cells for 1 hour. The supernatant was then removed and replaced with 0.5ml of the medium containing the viral plaques. These were incubated for 2 days at 27°C.

The cells were then pelleted by centrifugation for 5 minutes at 2000 rpm in a benchtop centrifuge. The pellet was washed in 0.5ml PBS. The cells were then resuspended in 0.5ml

0.5M NaOH to allow lysis and denaturation of the DNA. The solution was neutralised by the addition of 135μl of 7.5M ammonium acetate (pH 7.7). The denatured DNA was spotted onto a nylon (N+) membrane using a Biorad dot blot apparatus. The membrane had initially been equilibrated by brief soaking in 0.2M NaOH; 1M ammonium acetate. The wells were rinsed with 0.2M NaOH; 1M ammonium acetate, prior to spotting the DNA on the membrane. The membrane was finally rinsed in 4xSSC, sealed in a plastic bag and fixed for 5 minutes a side by ultraviolet trans-illumination. The membrane was soaked in prewarmed pre-hybridization buffer (2.2.12) at 42°C for 40 minutes. The gene derived probe, which was prepared as described in 2.2.10, was then added to the membrane. The membrane was then washed and autoradiographed as described in 2.2.10.

3.2.15 Preparation of recombinant baculovirus stocks.

A recombinant virus plaque (see 3.2.14) was used to infect $3x10^5$ cells for 4 days at 27°C. The supernatant was collected and used to infect $1.8x10^6$ cells. The supernatant was diluted to 1ml with Grace medium (FCS^+ and $antibiotic^+$) and left for 90 minutes at room temperature and then diluted to 3ml with Grace medium (FCS^+ and $antibiotic^+$). This was left for 4 to 5 days at 27°C. The supernatant derived from this, was used to infect a monolayer of $1x10^7$ cells in a sterile $80cm^2$ tissue culture flask. The supernatant was diluted to 6ml with Grace medium (FCS^+ and $antibiotic^+$) and left for 90 minutes at room temperature, at which time it was diluted to 20ml with Grace medium (FCS^+ and $antibiotic^+$) and left for 4 to 5 days at 27°C. The supernatant was harvested and titrated as described in section 3.2.13.

3.2.16 *In vivo* radiolabelling of recombinant baculovirus infected cells.

Cells were seeded at a density of $1.8x10^6$/well and infected for 23 hours at 27°C at an initial m.o.i of 10 with recombinant baculovirus stocks. The medium was then removed and the

cells washed once with 2ml of Eagle's medium lacking methionine. The cells were then incubated for 60 minutes at 27°C in 1ml methionine free Eagle's medium. A final concentration of 12μCi/ml S^{35}-met was added and the incubation continued for a further 3 hours at 27°C. The cells were detached from the surface and washed in PBS. The cells were resuspended in PBS, and lysed in 2xPSB and electrophoresed by 12% discontinuous SDS PAGE (Laemmli, 1970). The gel was stained and fixed as described in 3.2.7, followed by exposure to Amplify™ for 25 minutes. The gel was then finally dried and autoradiographed.

3.2.17 Western blot analysis of baculovirus infected cells.

0.2ml of 4 day post infected supernatants from single plaques infections of $3x10^5$ *sf9* cells were used to infect $3x10^5$ cells. Otherwise, stocks of known titre were used to infect the cells at a m.o.i of between 1 and 10. The infections were left for 90 minutes at room temperature, and then diluted to 0.5ml with Grace medium (FCS^+ and $Antibiotic^+$) and left for 2 to 4 days at 27°C. The cells were harvested by first replacing the medium with PBS, and then detaching the cells with a rubber syringe tip. The cells were washed twice with PBS. The cells were then resuspended in 100μl PBS. 10μl of the cell samples were mixed with an equal volume of 2x PSB and vortexed for 1 minute. The samples were then boiled for 3 minutes and sonicated for a further 5 minutes. The samples were briefly centrifuged in a benchtop centrifuge to remove any insoluble material. The supernatants were loaded onto a discontinuous 12% SDS polyacrylamide gel and electrophoresed.

For the transfer, the gel was placed on top of a Hybond-C (Amersham) nitrocellulose membrane. This was then sandwiched between to pieces of 2MM filter paper. All of these components had been presoaked in the transfer buffer (25mM Tris.HCl; 50mM glycine and 20% methanol; pH 8.3), for twenty minutes. The transfer was carried out for 60 to 90 minutes using a Trans-blot cell (Biorad) as specified. Rainbow marker (Biorad) was included

on the gel to control for the efficiency of transfer. The gel was stained after the transfer to further evaluate the efficiency of the transfer (3.2.7). The membrane was then soaked in PBS, followed by a 60 minute soaking in blocking buffer (1% fat-free milk powder in PBS) to prevent non-specific antibody binding to the membrane. Anti-guinea pig α-AHSV-4 serum (obtained from OVI) diluted 1:50 to 1:100 in blocking buffer was added to the membrane and incubated overnight at room temperature with gentle agitation. The membrane was then washed 3 times at 5 minutes each in 30ml volumes of PBS; 0.05% tween 20 (v/v), and then once more in PBS for 5 minutes. To label the primary antibody, the membrane was covered with a 1:1000 dilution of a protein A-peroxidase conjugate (Sigma) in blocking buffer for 60 minute at room temperature with gentle agitation. The washes were repeated as described before. The protein A-peroxidase conjugate was made visible by the addition of the peroxidase substrate (0.05% 4-chloro-1-naphthol [Sigma]; 0.5% H_2O_2; 17% methanol; 1 x PBS). After the appearance of the stain, the membrane was rinsed in PBS and air dried.

3.3 Results.

3.3.1 Tailoring of the segment 3 cDNA for baculovirus expression.

The strategy used for cloning the segment 3 genes of AHSV-3 and AHSV-9 resulted in the introduction of GC tails flanking the cloned genes. The presence of GC tails has a deleterious effect on the gene expression in the baculovirus system, as a high GC content has been shown to bring about reduced expression (O'Reilly *et al.*, 1992). It was therefore necessary to remove the tails.

The segment 3 gene of BTV was tailored for expression by limited exposure of the termini of the cDNA to *Bal31* exonuclease to remove unwanted nucleotides (Inumaru *et al.*, 1987). The shortened cDNA's were then characterised to identify products of the correct length for expression. Another approach for removing the tails which allows the termini to be defined in a more exact way, is one involving PCR (Nel and Huismans, 1991). The method involves designing PCR primers flanking the portion of the gene to be amplified. With this approach, convenient restriction sites can be designed at the 5' ends of the primers to facilitate cloning. PCR does have a higher error rate than other polymerases and thus will allow a reasonable probability that a gene the size of VP3 will incorporate errors. However, this method has been successfully used to express the even larger VP2 gene of AHSV-3 (Vreede and Huismans, 1994).

For the PCR approach, two primers SI5 and SI3, were designed to recognise the 5' and 3' ends of the open reading frames of both serotypes, respectively. Due to the high sequence conservation of the genes, (see section 2.3.3) it was possible to design primers that were complimentary to the termini of both serotypes with no mismatches. Non-coding bases were excluded from the 5' end as they could interfere with translation initiation (O'Reilly *et al.*, 1992). The primers were anchored with *BglII* restriction sites to facilitate cloning into the

baculovirus transfer vector pACUW3, which offers the choice of cloning foreign genes into a *BamHI* or *BglII* site (Weyer and Possee, 1991). The serotype 3 recombinant has an internal *BglII* site, but its position, 0.9Kb from the 5' end, should make the isolation of the full gene, by means of partial digestion, a viable option.

The resultant PCR products, after amplifying the cDNA templates of AHSV-3 and AHSV-9, both migrated to the anticipated size of 2.8kb (shown here in fig 3.1 is the serotype 9 PCR product [lane 6]), just above the 2.32Kb band of the molecular weight standard (lane 1). The PCR product migrates slightly faster than the original cDNA clone, excised by *PstI* digestion directly out of pBR322, due to the absence of G/C tails and non-coding bases (lane 5). Further confirmation that the PCR products were of segment 3 origin came from the results of *HindIII* digests of the PCR products. Both serotypes share a conserved *HindIII* site approximately 0.7Kb from their 3' termini which is shown here for the AHSV-3 cDNA recombinant (lane 4), where the smallest fragment corresponds to the $\pm$0.7Kb fragment of segment 3. The band migrating just above this corresponds to the $\pm$0.75Kb *PstI/HindIII* vector-specific band. The next band (lane 4) running close to the 2.3Kb band corresponds to the *PstI/HindIII* segment 3 fragment. The band migrating above this, is the remainder of pBR322. The AHSV-3 and AHSV-9 segment 3 PCR products (lanes 2 and 3 respectively) have *HindIII* restriction patterns that are similar to the segment 3 specific bands excised from pBR322 after *PstI/HindIII* digestion (lane 4), except that the 700bp fragments do move noticeably faster. This is probably due to the absence of the G/C tails, and most of the non-coding bases.

The serotype specificity of the PCR products was confirmed by *BglII* digests (results not shown) since the AHSV-3 clone has an internal *BglII* site which is absent from AHSV-9.

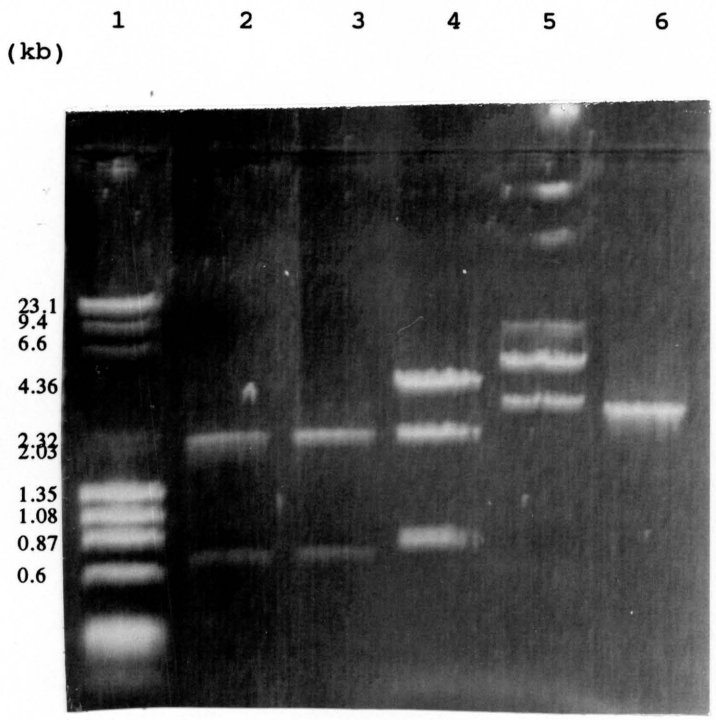

fig 3.1 Ethidium bromide stained 1% agarose gel of PCR products of AHSV-3 and AHSV-9 segment 3 gene. Lane 1 is molecular weight marker II and *HaeIII* digested ϕX 174 DNA (Boehringer Mannheim). Lanes 2 and 3 are PCR products of AHSV-3 and AHSV-9 respectively, digested with *HindIII*, whereas Lane 6 shows the AHSV-3 PCR product, undigested. Lanes 4 and 5 are the cDNA clone of AHSV-9 cloned into pBR322, digested with *HindIII* and *PstI* (lane 4) and with *PstI* alone (lane 5).

Prior to baculovirus expression of the tailored segment 3, it was first necessary to check the expression of these genes in alternate systems to ensure that no nonsense errors had been introduced into the genes by the PCR approach.

3.3.2 Expression of AHSV-9 VP3 as a bacterial fusion protein.

Since the segment 3 genes were tailored by PCR, there was the possibility that nucleotide mis-incorporations could have occurred. This may have resulted in the introduction of nonsense mutations. In order to determine if the normal full length open reading frames of VP3 were maintained, the AHSV-9 VP3 gene was selected to be first expressed in a bacterial expression system. In addition to this, many unsuccessful attempts have been made in other laboratories to express the full length VP3 gene of AHSV (Huismans, pers. comm.), and to therefore express this protein in an alternative system such as a bacterial expression system could shed some light on the general expression of this protein.

Two different segment 3 PCR products of AHSV-9 were cloned. The first product (PCR-I) was cloned into the *BglII* site of the baculovirus transfer vector pACUW3 (Weyer and Possee, 1991) by digesting the termini of the PCR product with *BglII*, and the second (PCR-II), by C-tailing the PCR product, and then cloning it into the G-tailed *PstI* site of pBR322. PCR-I and PCR-II were then subcloned into the *BamHI* site of pGEX-2T by *BglII* terminal digestion. This will create an in-frame fusion of the N-terminal portion of VP3 with the 26kD Guanosyl S-transferase (GST) protein of *Schistosoma japonicum* which is under the control of the IPTG inducible, tac promoter (Smith and Johnson, 1988). The fusion proteins have the capacity to be purified due to the presence of the N-terminally located GST component. The pGEX vector series, pGEX1, pGEX-2T and pGEX-3X allows a fusion protein to be constructed in each of the three possible reading frames, respectively. In addition to being cloned in pGEX-2T, PCR-II was also cloned into pGEX1 and pGEX-3X.

The recombinants were first characterized by restriction mapping to ensure they were full length, and to determine the orientation. The clones were induced with IPTG and analyzed by Coommasie stained SDS/PAGE (fig 3.2). The induction with IPTG was confirmed by the presence of the 27kD protein in all the induced samples of the parental vectors, pGEX1, pGEX-2T and pGEX3X (lanes 14, 10 and 6 respectively). The induced protein is absent from controls not treated with IPTG (lane 13, 9 and 5). Of the two PCR products, only the clone containing PCR-II expressed correctly. PCR-II cloned into pGEX-2T shows a novel protein at approximately 116kD (lane 8). This band is at approximately the position expected for a correct fusion protein. This band is absent from the un-induced control (lane 7). As expected, the PCR-II product cloned into pGEX1 shows a truncated protein (lane 10), absent from its un-induced control (lane 9) which is of a similar size to the non-recombinant GST protein (lanes 6, 10 and 14). The pGEX-3X PCR-II recombinant does not appear to be induced (lane 12). The reasons for this were not investigated. The PCR-I clone, on the other hand was not expressed as a protein of the expected size. The induced PCR-I recombinant cloned into pGEX-2T, showed a novel protein (lane 2), which was not evident in the un-induced recombinant (lane 1), or parental vector (pGEX-2T) induced controls (lanes 10). The size is approximately 11 kD larger than the 27kD GST non-fusion protein (lane 10), but nevertheless, smaller than the fusion protein produced from PCR-I in the same vector (lane 8). The possibility that a deletion or insertion occurred at the cloning junction site is unlikely, because this same situation is present in two independent clones (data not shown).

The most likely explanation for the truncated GST fusion protein, is that a nonsense error was introduced into the PCR-I product by PCR. These results also demonstrated that a full length VP3 can be expressed in the form of a fusion protein, in a bacterial expression system. Attempts to purify the protein by affinity chromatography were unsuccessful due to the high insolubility of the fusion protein. The protein was found to be associated with the cell debris during the purification process (data not shown).

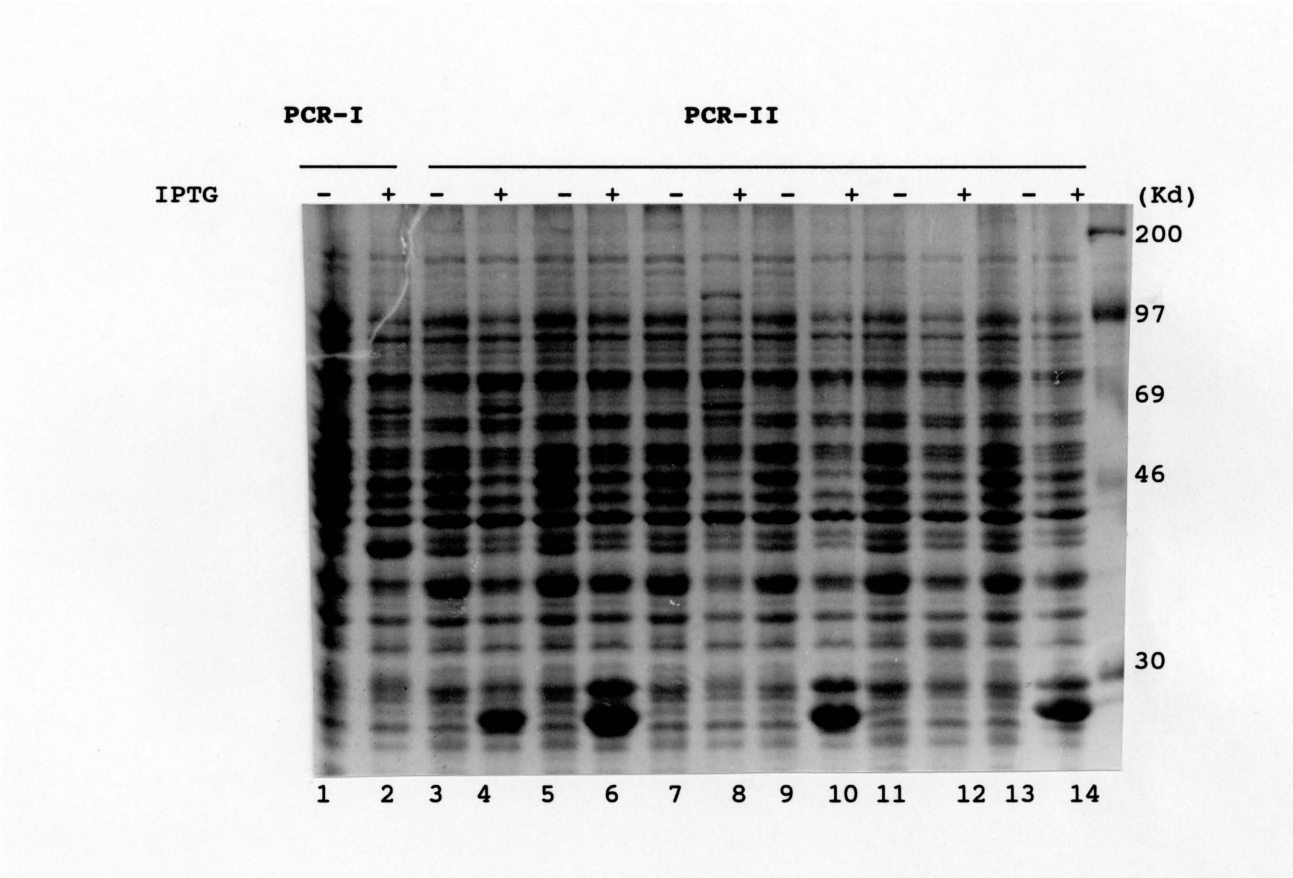

fig 3.2 Coommassie stained 12% PAGE analysis of VP3 of AHSV-9 expressed as a GST fusion protein in *E. coli*. The VP3 being expressed in pGEX-2T is shown in lanes 1, 2, 7 and 8, and in pGEX-1, lanes 11 and 12. The VP3 expressed in pGEX-3X is shown in lanes 3 and 4. The non-recombinant parental vectors are shown in lanes 5 and 6 (pGEX-3X); lanes 9 and 10 (pGEX-2T); and lanes 13 and 14 (pGEX-1). The sizes (in kD) are indicated on the right hand side of the figure.

3.3.3 *In vitro* translation of AHSV-3 segment 3 and the immune precipitation of the resulting product.

Another approach to assessing whether a full length gene product is being expressed is to express the gene in an *in vitro* system such as a cell free lysate derived from rabbit reticulocytes where the gene of interest is expressed off exogenously added mRNA.

In order to do this, the gene was first cloned into a transcription vector, in this case pBS, which consists of the phage T7 and T3 promoters. Segment 3 specific mRNA could then be expressed *in vitro* using phage T3 or T7 RNA polymerase. The *in vitro* synthesized mRNA can then be used as a template for the expression of the VP3 protein in the cell lysate.

The termini of the AHSV-3 segment 3 PCR product has *BglII* sites which were introduced by the primer sequences during amplification. Since there is also an internal *BglII* site 0.9Kb from its 5'end, the fragment could only be cloned in a full length form by partial *BglII* digestion. The PCR product was first cloned by the C-tailing method into pBR322 (see 3.2.3). The partial digest was then done by performing a serial dilution of the restriction enzyme. The results were analyzed by 1% agarose gel electrophoresis (see fig. 3.3). The desired 2.8Kb fragment, representing the full length segment 3 gene, appears in lanes 4 to 7. It is at a maximum in lane 7. In lanes 2 and 3, the digest is almost complete, where only 3 bands are predominantly visible. These are the 4.36Kb pBR322 fragment, and the two segment 3 products of 1.9 and 0.9kb. A minor partial product 0f 6.26Kb is evident at the lowest enzyme dilutions (lanes 2 and 3) representing the vector band plus 1.9Kb of the segment 3 gene, showing that digestion was not complete. The other partial fragment of 5.26Kb (vector plus the 0.9Kb fragment) is partially masked by the 4.36kB vector band (lanes 2 and 3), but becomes more evident as its amount increases (lanes 4 to 7). The digests represented in lanes 8 and 9 appear very similar to the undigested control (lane 10), demonstrating that this enzyme concentration does not support efficient digestion of the

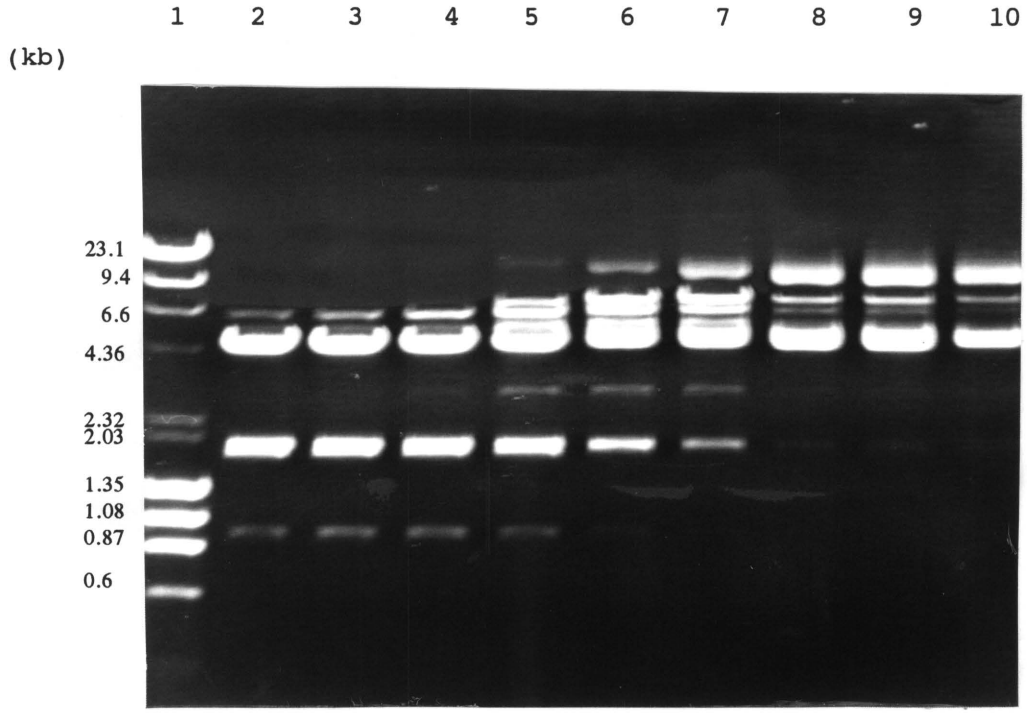

fig 3.3 1% agarose gel electrophoresis showing the *BglII* partial digestion series of the AHSV-3 PCR product cloned into pBR322. DNA molecular weight markers are shown in lane 1, with the sizes indicated on the left of the figure. The partial digest series is shown in lanes 2-9, with decreasing amounts of enzyme. Undigested plasmid is shown in lane 10.

plasmid under these conditions. The conditions represented in lane 7, were scaled up to yield large amounts of the 2.8Kb fragment which was then was directly cloned into the *BamHI* site of the pBS transcription vector, in the orientation required for transcription off the T7 promoter, as determined by restriction mapping. Before this construct was used as a template for mRNA synthesis, it was linearised at a site downstream of the cloned insert by digestion with *XbaI* which is present in the multiple cloning site (MCS), downstream of the gene, but absent from the gene. The mRNA from the T7 transcription reactions was analyzed by 1% agarose gel electrophoresis (results not shown), and then used in the *in vitro* translation reactions.

The results of the *in vitro* translation are presented in figure 3.4. A protein synthesized by the *in vitro* translation of VP3 mRNA (lane 2) co-migrates with the VP3 of S^{35}-met labelled proteins of CER cells infected with AHSV-3 (lane 1). The protein was not only the same size as VP3 but could also be immune precipitated with a AHSV-4 specific serum (lane 3). Such an inter-serotype reaction would be anticipated since VP3 has been identified as a group specific antigen (Huismans and Erasmus, 1981). The presence of smaller bands in the *in vitro* translated VP3 lane (lane 3) may either be degradation or premature termination products of VP3, as they are precipitated with the band of the same mobility as the authentic VP3. A similar profile of smaller bands was obtained for the *in vitro* translated VP3 of BTV (Liu *et al.*, 1992). In addition to this, these novel bands were not present in the *in vitro* translated VP7 control (lane 4) (mRNA provided by S. Maree, dept. Genetics, U.P), or the negative control reaction (lane 6), which had no exogenously added RNA, further supporting their being of VP3 origin.

Since this protein migrated at exactly the same position as the authentic VP3 of virus-infected cells, and because it was strongly immune precipitated by AHSV specific serum, it could be concluded that this was an authentic, full-sized VP3 protein. The AHSV-3 VP3 gene was therefore used for expression in the baculovirus system.

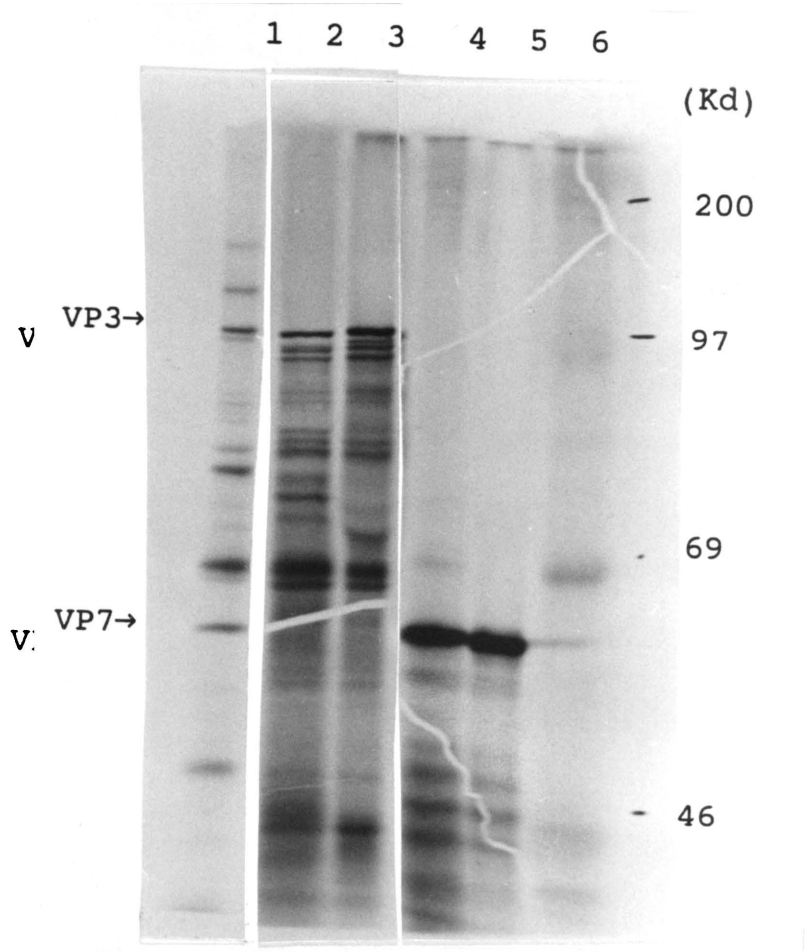

fig 3.4 Autoradiogram of *in vitro* translated VP3 separated by 12% PAGE. Lane 1 shows CER cell extracts infected with AHSV. Lane 2 is the *in vitro* translated VP3. Lane 3 is the translated VP3, immune precipitated with αAHSV-4 serum. Lane 4 shows a VP7 translated control, with the immune precipitated product shown in lane 5. Lane 6 is a a control where no exogenous mRNA was added. VP3, VP7 and the size markers are indicated on the figure.

3.3.4 Construction of a VP3 baculovirus recombinant.

The approach followed to construct a baculovirus recombinant that expresses the VP3 protein involves the co-transfection of a plasmid containing the segment 3 gene cloned downstream of the promoter driving the expression of the non-essential polyhedrin gene, which is flanked by part of the polyhedrin gene sequence, together with baculovirus DNA linearised at a unique site. A double crossover event between the polyhedrin gene of the baculovirus genome and the polyhedrin sequences in the transfer vector restores the baculovirus DNA to a circular form, thus allowing the production of viable virus with the foreign sequence replacing the polyhedrin gene. This approach using linearised baculovirus DNA, has been shown to produce a higher proportion of recombinants than if the baculovirus genomic DNA was transfected in a circular form (Kitts *et al.*, 1990).

The AHSV-3 segment 3 PCR product was cloned by a partial *BglII* digest (see 3.2.5) into the *BamHI* site of the baculovirus transfer vector, pACUW3 (Weyer and Possee, 1991). This positions the segment 3 gene under the control of the polyhedrin promoter. AcRP23 *lacZ* baculovirus DNA was used as the parental virus DNA. This virus DNA can be linearised at a unique *Bsu 36i* site within a copy of the *lacZ* gene, at the polyhedrin locus of the baculovirus genome (Kitts *et al.*, 1990). The linearised AcRP23 *lacZ* DNA was co-transfected together with the AHSV-3 PCR pACUW3 recombinant into insect cells.

A plaque assay of the transfection supernatants was used to identify recombinants. Recombinants can be identified as white plaques, since the *lacZ* gene is replaced by the foreign gene during the recombination event. The titration of the supernatant obtained from the co-transfection yielded unexpected results when compared with a transfection control lacking transfer vector. Both yielded similar plaque profiles. Both samples contained white plaques in addition to the blue plaques (non-recombinant) although there was a higher proportion of white plaques in the co-transfection. This suggests that the *lacZ* marker gene

had been mutated or deleted within the viable virus, via a process independent of the presence of the transfer vector. This will make selection of the recombinant virus difficult as the selection process is based on the presence of a white plaque phenotype being introduced into the virus genome via the recombination event with the transfer vector.

Several of the white plaques resulting from the co-transfection, were screened for the presence of the segment 3 gene in a Dot Blot assay using the full length segment 3 gene as a probe. Three out of ten white plaques gave a positive signal (data not shown).

3.3.5 PAGE detection of baculovirus expressed AHSV-3 VP3 by Coomassie Blue staining and *in vivo* radiolabelling of Baculovirus recombinants.

The Dot Blot hybridization results proved that the VP3 gene had been successfully incorporated into the baculovirus genome. To determine whether the gene was expressed, the lysates of insect cells infected with the AHSV-3 VP3 baculovirus recombinant were examined by coommassie stained SDS PAGE. A protein the size of VP3 (lane 5 fig 3.5) was expressed at 48 hours post infection to similar levels as the LacZ protein of the parental AcRP23 *lacZ* virus (lane 3, fig 3.5). There are proteins of a similar size, at this time in both AcRP23 *lacZ* (lane 3) and in the mock infected control (lane 1) suggesting that they are of cellular origin. At 96 hours post-infection the protein is more clearly evident when compared with the AcRP23 *lacZ* recombinant (lane 4), as the cellular protein, which runs at a similar position to the VP3 band, is greatly reduced.

However, subsequent to this experiment, when high titre stocks of this virus were produced, the levels of this recombinant protein declined. The protein was still evident, but only at 96 hours post infection) (data not shown) once the cell proteins had declined to a significant level.

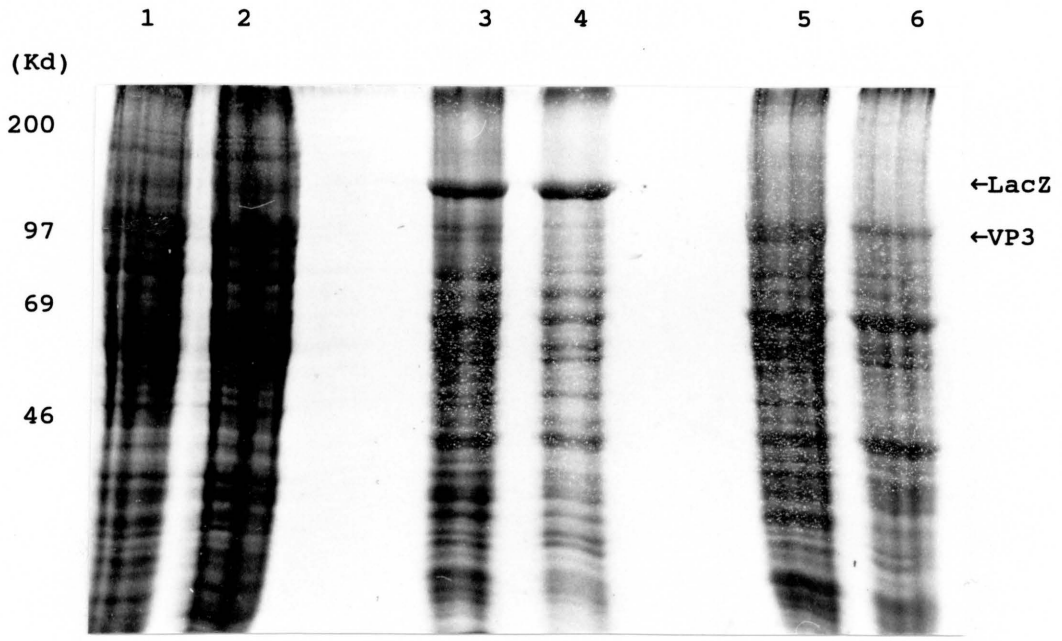

fig 3.5 Coomassie stained PAGE analysis of insect cells infected with baculovirus recombinant. Lanes 1 and 2 are a mock infected control at 48 hpi 96 hpi respectively. Lanes 3 and 4 are the "wild type" AcRP23 *lacZ* parental virus control, at 48 and 96 hpi respectively, while lanes 5 and 6 are the potential VP3 baculovirus at 48 and 96 hpi respectively. The potential VP3 and LacZ bands are indicated on the figure.

Since the VP3 gene is cloned under the polyhedrin very late promoter, it's expression should be detectable by radio-labelling insect cells at approximately 20hpi, where the expression of this promoter is at high levels (O'Reilly *et al.*, 1992). Insect cells were infected with the potential VP3 recombinant, and at the time just prior to very late gene expression, the cells were starved of methionine by incubation in methionine free medium. S^{35}-methionine was then added to the medium, so that the newly synthesized protein would incorporate the labelled methionine residue. The cell extracts could then be analyzed by SDS PAGE and autoradiography.

The results of the *in vivo* radiolabelling (figure 3.6) clearly demonstrate the presence of a novel band in the VP3 AHSV-3 baculovirus recombinant (lane 3) co-migrating with the native VP3 protein of radio-labelled CER cells infected with AHSV (lane 4). This band is absent from both an NS3 baculovirus recombinant (provided my M. Stolts) (lane 2) and a mock infected cell control (lane 1).

It was therefore evident that a unique protein of the size anticipated for native VP3, was being expressed. To further confirm that the protein is VP3, western blots were performed using AHSV specific serum.

3.3.6 Western Blot analysis.

To confirm that the novel protein that was expressed is of AHSV origin, a Western blot was performed on a whole extract of insect cells carrying the potential VP3 baculovirus recombinant. The results of the Western Blot are shown in figure 3.7. The two round plaque purified recombinant (lanes 2 and 3) show a band which reacts to the serum directed against AHSV-4, at the expected size for native VP3, when compared to the S^{35}-methionine labelled AHSV infected CER cells (lane 5). An *in vivo* labelled control of AHSV infected CER cells was also included as the unlabelled sample of AHSV infected CER cells gives a

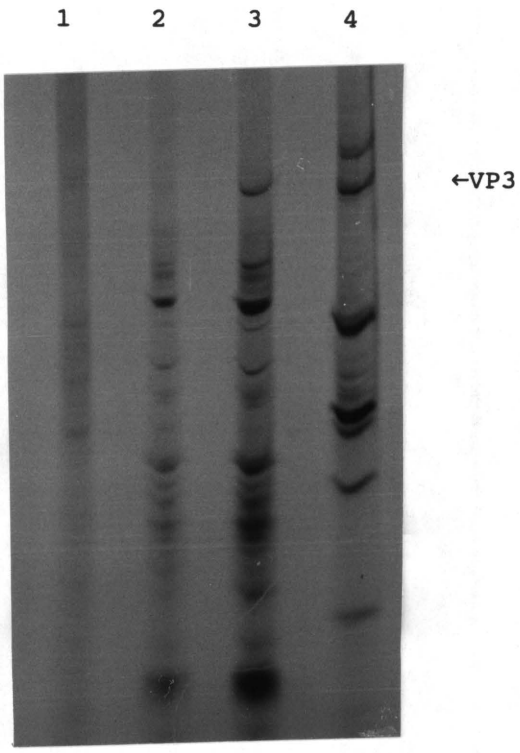

fig 3.6 Metabolic labelling of insect cells expressing the baculovirus recombinant. Lane 1 is a mock infected control. Lane 2 is a baculovirus control expressing the AHSV NS3 protein. Lane 4 is the VP3 recombinant, while, lane 5 is a control of radiolabelled AHSV-infected CER cells. The VP3 protein is indicated on the figure.

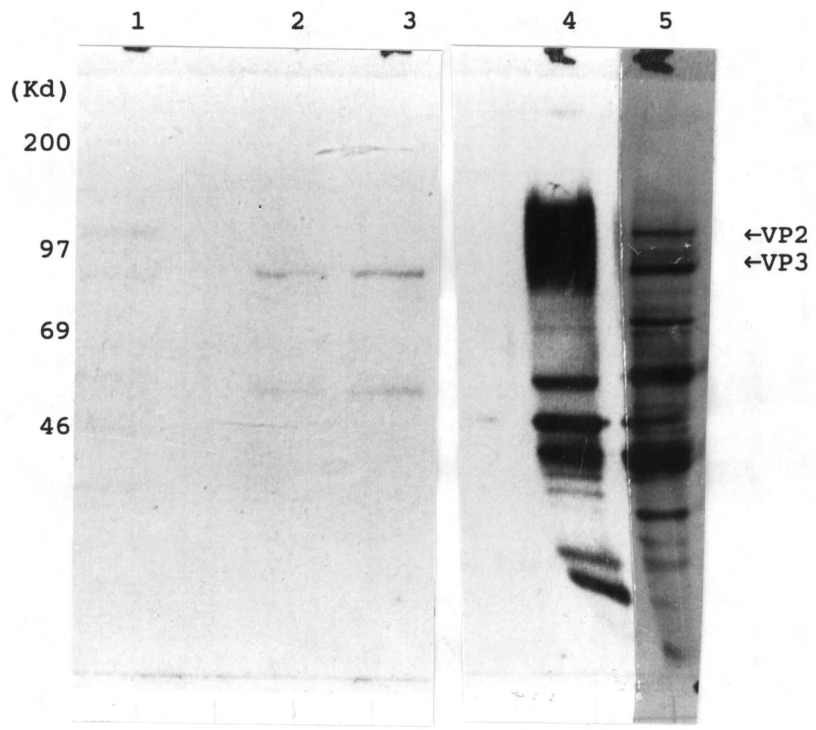

fig 3.7 Western Blot analysis of insect cells expressing the VP3 baculovirus recombinant. Lane 1 is the AcRP23 *lacZ* parental virus control, while lanes 2 and 3 are the VP3 recombinants. Lane 4 and 5 are CER cells infected with AHSV. The sample in lane 5 has been metabolically labelled with S^{35}-methionine. The molecular weights, VP2 and VP3 are indicated on the figure.

smear in the region of the native VP3 protein (lane 6). The AcRP23 *lacZ* control has a protein of about the same size as native VP3 as well as the LacZ band of the "wild type" which reacted very weakly with the serum. This reaction could however be clearly distinguished from the much stronger reaction with VP3.

3.4 DISCUSSION.

The VP3 protein of AHSV-3 was successfully expressed in the baculovirus expression system. The levels were initially comparable to the LacZ expression of the parental baculoviral vector AcRP23 *lacZ*, but the levels declined after the production of high titre stocks for unexplained reasons.

The protein was shown to be of the correct size for native VP3 as it co-migrated with the VP3 of purified AHSV virions and the VP3 derived from AHSV infected CER cells, on SDS PAGE gels. The protein was also the same size as that synthesized *in vitro* in the rabbit reticulocyte lysates, giving a strong indications that no modifications occur in insect cells. The authenticity of the AHSV-3 VP3 protein was further confirmed in Western blots by its reaction with AHSV-4 serum. VP3 is not serotype specific and it can be assumed that it will cross-react between different serotypes. This has also been shown to be the case in the case of BTV (Inumaru *et al.*, 1987).

The baculovirus system usually yields high levels of expression of heterologous genes off its non-essential polyhedrin and P10 promoters (O'Reilly *et al.*, 1992; Miller, 1988). However, in the case of the AHSV-3 VP3 baculovirus recombinant the levels of VP3 expression declined and *in vivo* metabolic labelling was required to unequivocally demonstrate its presence. These results do not seem to be as a result of the orbivirus VP3 protein as reasonably high levels in the case of BTV have been demonstrated on Coommassie stained gels (Le Blois and Roy, 1993) and subsequent high level expression of AHSV-9 VP3 has been achieved in our laboratory using an alternative expression system (Maree *et al.* submitted). These differences in expression are unlikely as a result of differences between the serotypes, since the protein is highly conserved at the primary sequence level (see chapter 2).

A factor contributing to the low levels may have been the different approaches followed to tailor the genes for expression. The expressed VP3 described here was generated by a PCR approach from the original cDNA template which served to get rid of the G/C tails which were introduced as part of the cDNA synthesis strategy. This method was first described for the baculovirus expression of the EHDV NS1 gene (Nel and Huismans, 1991). A similar approach was followed for the expression of the AHSV-9 PCR product (Maree *et al* submitted), but the open reading frame generated by the PCR was largely replaced with the original open reading frame derived from the cDNA by using conveniently placed restriction sites located within the gene. This would eliminate the potential errors introduced by the PCR approach. The potential for the introduction of errors resulting from PCR must be quite large as a PCR product expressed as a AHSV-9 GST::VP3 fusion protein was found to be truncated. The most plausible explanation for this truncation was the introduction of an in-frame stop codon resulting from the PCR reaction. This is also strongly supported by the demonstration that a newly generated GST::VP3 fusion protein was not truncated, and migrated to a size anticipated for the full length GST::VP3 fusion protein. Less obvious mutations could potentially alter the expression of the protein leading to reduced levels.

Since, the original expression of the AHSV-3 VP3 was easily detectible at 48 hours post infection, and the levels only declined after making high titre stocks from the original supernatant, it is possible that the reduced expression levels of the VP3 protein may have been ascribed to an instability in the parental baculovirus, AcRP23 *lacZ*. It was found that transfection of the linearised AcRP23 *lacZ* DNA, in the absence of transfer vector, yielded a small proportion of white plaques in addition to the expected blue plaques. The blue plaques could have been from incomplete linearization of the virus DNA. However, in the case of the white plaques, re-circularization may have occurred that aberrantly affected the reading frame of the *lacZ* gene. Kitts *et al*. (1990) found in a similar situation when the genome based *lacZ* gene was located in the P10 promoter, that as many as 60% of the

plaques resulting from a transfection experiment were non-recombinant but failed to express the *lacZ* gene.

White plaques may also have been the result of deletions of the *lacZ* gene, or in the case of the AHSV-3 segment 3 recombinant, deletion of the heterologous gene. This is supported by findings where the VP3 gene of 3/15 of the recombinant viruses was lost after titration of the original recombinant virus stock. This was demonstrated by dot blot hybridization (result not shown). The possibility that false plaques were accidentally picked was checked by infecting cells derived from the plaque and looking for cytopathic effect against an uninfected cell control. It seemed therefore, that the most likely explanation for the reduced levels of expression of the AHSV-3 VP3, was an instability in the parental AcRP23 *lacZ* virus. It should be noted here that the VP2 gene of AHSV-3 was also expressed at low level in the baculovirus system (Vreede and Huismans, 1994). The AcRP23 *lacZ* was also the parental vector used in that study.

The baculovirus expressed AHSV-3 VP3 protein was highly insoluble, since it pelleted through 10-40% sucrose gradients, after a 30 minute centrifugation at 48000rpm. This insolubility was not alleviated by the addition of 0.5M NaCl (data not shown) suggesting that it is an inherent property of the protein, and unlikely to be the result of non-specific interactions with cellular proteins or nucleic acids. The GST::VP3 fusion protein of AHSV-9 could not be purified as it was found to also be highly insoluble. It is therefore probable that AHSV VP3, when expressed in the absence of other orbiviral proteins, is quite insoluble.

Chapter 4. CONCLUDING REMARKS.

Attempts to express an AHSV VP3 have to date, not been successful (Huismans. pers. comm). Due to the importance of this protein as a potential scaffold on which to present the major immunogenic determinant, VP2 in the form of VLPs, and in understanding the basic biology of this virus, a study was undertaken to see if this gene could first of all be cloned as a full length copy, and then subsequently be expressed as a baculovirus recombinant.

Both the AHSV-3 and AHSV-9 segment 3 genes were cloned, and found to be full length with respect to their open reading frames. Sequence comparisons have not been done on this gene within this serogroup, as only the AHSV-4 segment 3 gene has been cloned and sequenced (Iwata *et al.*, 1992). As much as 16% of the open reading frames at the termini of the AHSV-3 and AHSV-9 were sequenced and compared with the AHSV-4 sequence. The results of the subsequent comparisons revealed that, as is found in other orbivirus serogroups, the segment 3 gene is highly conserved.

Since the AHSV-4 segment 3 gene could not be successfully expressed in the baculovirus system, we decided to investigate the expression of a different serotype of AHSV in the baculovirus system, and we also looked at the expression of this gene in different expression systems to test the general expressability of this protein. It was found that the AHSV-9 segment 3 gene could be expressed in a full length form, as a fusion protein, in a bacterial expression system. Expression of a full length AHSV-3 segment 3 gene was also achieved *in vitro*. Since it was shown that the gene was expressible, a baculovirus recombinant of the AHSV-3 segment 3 gene was produced. Initially the levels were easily detectable by coomassie blue staining of SDS polyacrylamide gels, but declined after the production of high titre stocks. The parental baculovirus strain used, AcRP23 *lacZ* was found to be unstable, which could therefore possibly explain the reduced levels of expression attained for this protein.

Subsequent studies using the BACMID™ baculovirus expression system have greatly overcome the problems of reduced expression levels encountered using the AcRP23 *lacZ* host. The approach allows the production of recombinant baculovirus genomic DNA within a bacterial host. Separate VP3 and VP7 baculovirus recombinants were constructed using this approach. The genes were cloned under the baculovirus polyhedrin promoter which is contained with a transfer plasmid. The genes were then introduced into an *E. coli* resident baculovirus genome by a transposition event via a helper plasmid. The baculoviral DNA, which is isolated directly from the bacterial cells, was then transfected directly into insect cells. The AHSV-9 VP3 baculovirus recombinant has been expressed in this system to very high levels (Maree *et al.*, 1997).

Recombinant baculoviruses of the VP3 and VP7 genes were used to co-infect insect cells. The derived cytoplasmic fraction of infected cells was banded on discontinuous sucrose gradients. Fractions predominant in both VP3 and VP7, as determined by SDS PAGE, were concentrated and examined by negative staining transmission electron microscopy. Particles similar to authentic AHSV cores and BTV core-like particles were evident. Decoration of the particles by VP7 monoclonal antibodies confirmed the presence of VP7 on the surface of these particles (Maree *et al.*, 1997). These results demonstrated that AHSV core-like particles could be synthesised using the baculovirus expression system.

It is still important, primarily for the production of safe recombinant vaccines, but also to achieve a greater understanding of AHSV biology, to investigate whether virus-like particles can be produced by expressing the four major AHSV structural proteins. This will require the simultaneous presence of all four proteins in the same insect cell. This can best be achieved by constructing baculovirus recombinants expressing two, or all four of these genes simultaneously. At present, work is in progress to construct dual expression vectors for the co-expression of VP3 and VP7 from the same infectious unit.

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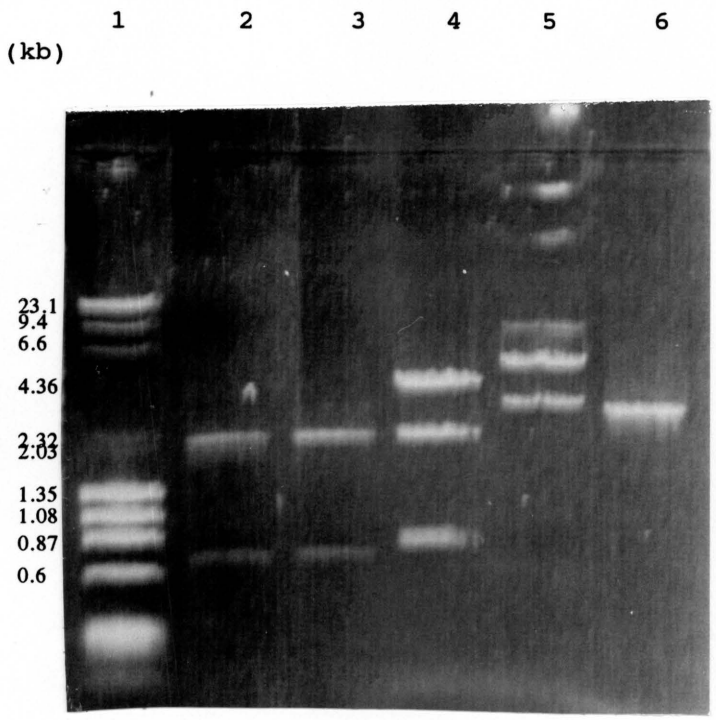

fig 3.1 Ethidium bromide stained 1% agarose gel of PCR products of AHSV-3 and AHSV-9 segment 3 gene. Lane 1 is molecular weight marker II and *HaeIII* digested ϕX 174 DNA (Boehringer Mannheim). Lanes 2 and 3 are PCR products of AHSV-3 and AHSV-9 respectively, digested with *HindIII*, whereas Lane 6 shows the AHSV-3 PCR product, undigested. Lanes 4 and 5 are the cDNA clone of AHSV-9 cloned into pBR322, digested with *HindIII* and *PstI* (lane 4) and with *PstI* alone (lane 5).

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