CHAPTER 3:CHARACTERIZATION OF THE VP6 PROTEIN OF AHSV

3.1 INTRODUCTION

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

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

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

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

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

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

3.2 MATERIALS AND METHODS

3.2.1 *IN VITRO* EXPRESSION

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

3.2.2 *IN VITRO* TRANSLATION

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

3.2.3 POLYACRYLAMIDE GEL ELECTROPHORESIS

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

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

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

3.2.5 PREPARATION OF COMPETENT CELLS BY THE DMSO METHOD

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

3.2.6 GENERATION OF RECOMBINANT BACMIDS IN DH10BAC CELLS BY TRANSPOSITION

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

3.2.7 ISOLATION OF COMPOSITE BACMID DNA

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

3.2.8 TRANSFECTION INTO *SPODOPTERA FRUGIPERDA* CELLS

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

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

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

3.2.9 INFECTION OF SF9 CELLS

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

3.2.10 VIRUS TITRATION BY PLAQUE ASSAY

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

3.2.11 INFECTION OF MONOLAYERS FOR VIRUS STOCKS AND PROTEIN

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

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

3.2.12 WESTERN IMMUNOBLOT

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

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

3.2.13 5' MODIFICATION BY PCR

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

3.2.14 AUTOMATED SEQUENCING

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

3.2.15 CLONING AND EXPRESSION IN A BACULOVIRUS SYSTEM

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

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

3.2.16 CLONING AND EXPRESSION USING A BACTERIAL SYSTEM

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

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

3.2.17 *IN VIVO* PROTEIN LABELLING

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

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

3.2.18 SUCROSE GRADIENT ANALYSIS

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

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

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

3.2.19 NI-NTA COLUMN PURIFICATION

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

3.2.20 GLYCOSYLATION ASSAY BY PAS STAINING

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

3.3 RESULTS

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

3.3.1 *IN VITRO* TRANSLATION OF VP6 mRNA

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

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

3.3.2 *IN VIVO* BACULOVIRUS EXPRESSION

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

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

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

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

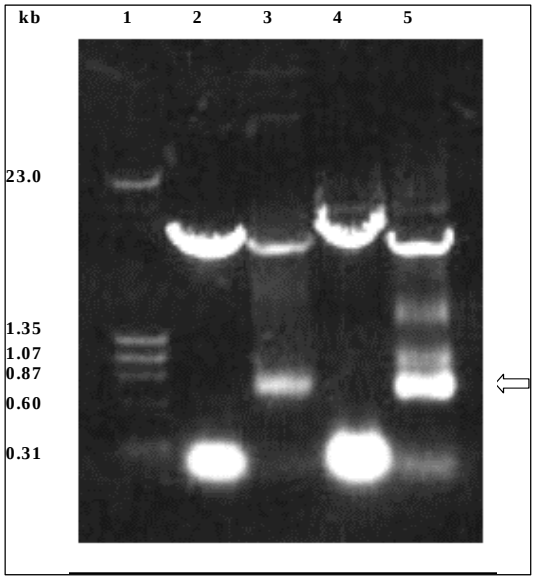

Figure 3.1 ***In vitro*** **transcription of the AHSV-3 VP6 gene (T3) and the AHSV-6 VP6 gene (T7).**

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

Lane 1: molecular weight markers;

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

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

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

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

The arrow indicates the mRNA of interest.

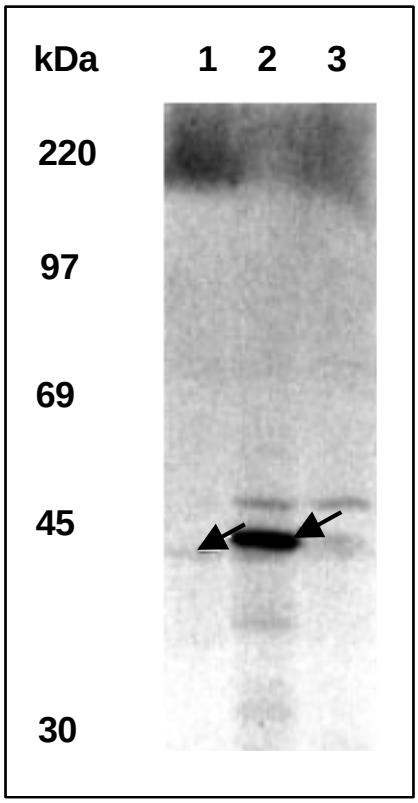

Figure 3.2 ***In vitro*** **translation of the transcription product of the genome segments encoding VP6 of AHSV-3 and AHSV-6.**

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

Rainbow marker sizes are indicated on the left in kDa.

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

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

lane 3: a negative control with no mRNA.

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

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

3.3.3 BACTERIAL EXPRESSION

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

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

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

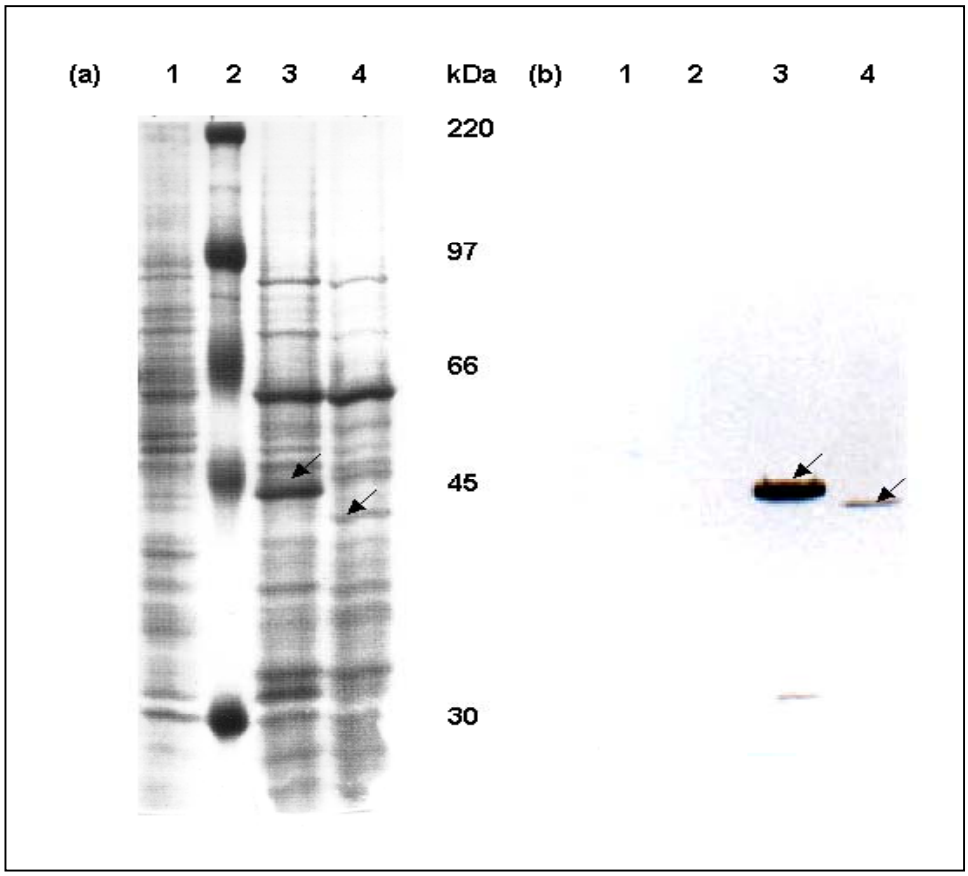

Figure 3.3 ***In vivo*** **expression and western immunoblotting of VP6 of AHSV-3 and AHSV-6.**

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

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

lane 2: Rainbow marker;

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

In b) lane 1: mock infected Sf9 cells,

lane 2: wildtype pFASTBAC infected Sf9 cells;

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

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

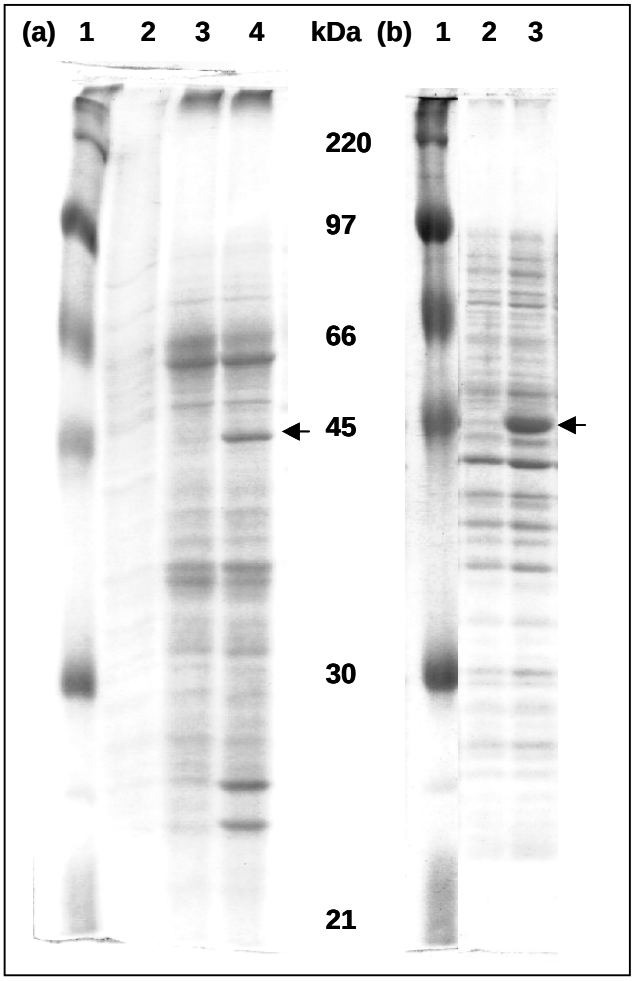

Figure 3.4 Comparison of expression of AHSV-6 VP6 in insect and bacterial cells.

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

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

In a) lane 2: mock infected Sf9 cells;

lane 3: wildtype vector infected Sf9 cells;

lane 4: recombinant VP6 baculovirus infected Sf9 cells.

In b) lane 2: control bacterial cells and

lane 3: bacterially expressed VP6.

The arrow indicates the VP6 protein.

3.3.4 WESTERN IMMUNOBLOT ANALYSIS

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

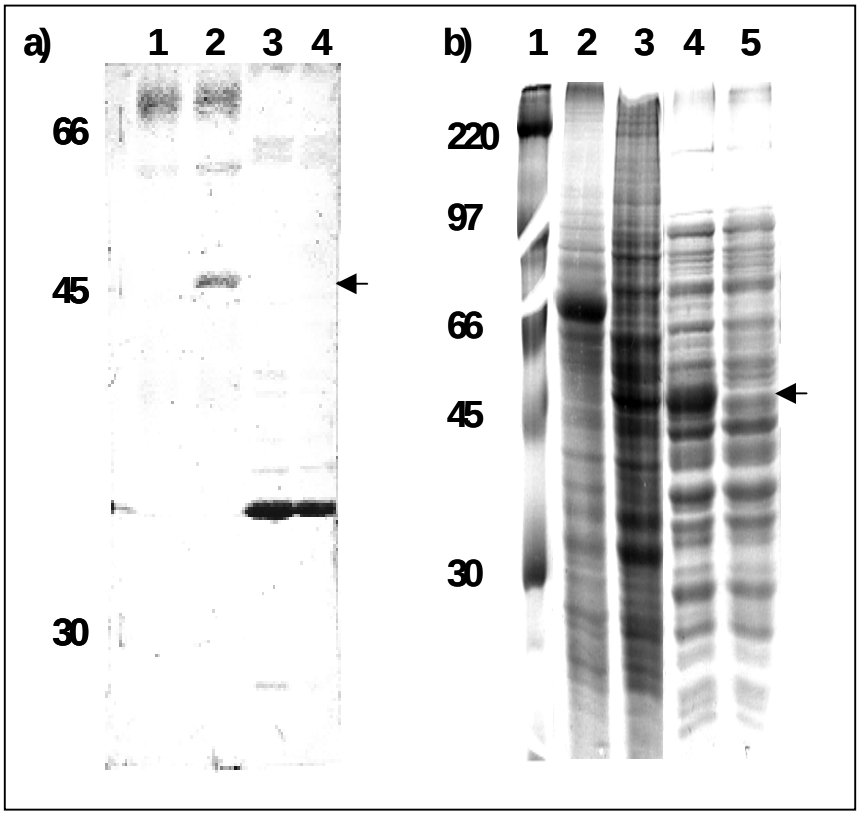

Figure 3.5 Immunological screening of baculovirus and bacterial expressed VP6.

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

lane 1: wildtype baculovirus infected Sf9 cells,

lane 2: baculovirus expressed VP6,

lane 3: bacterially expressed VP6,

lane 4: control bacterial cells.

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

lane 1: Rainbow marker

lane 2: wildtype baculovirus infected Sf9 cells,

lane 3: baculovirus expressed VP6,

lane 4: bacterially expressed VP6,

lane 5: control bacterial cells.

3.3.5 PROTEIN SOLUBILITY STUDIES

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

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

3.3.6 PROTEIN PURIFICATION

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

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

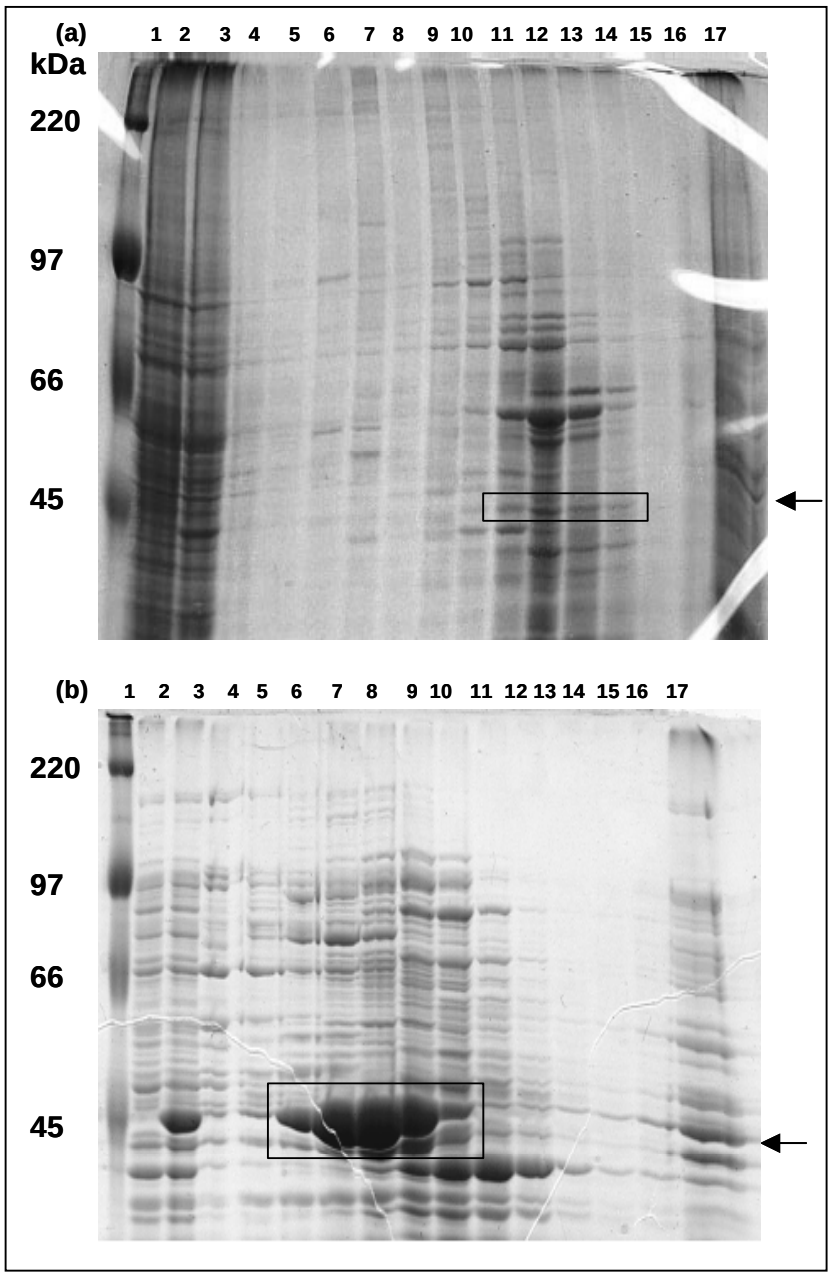

Figure 3.6 Sucrose gradient fractionation of a) baculovirus expressed VP6 and b) bacterially expressed VP6.

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

In both a) and b)

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

3.3.7 GLYCOSYLATION ASSAY BY PAS STAINING

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

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

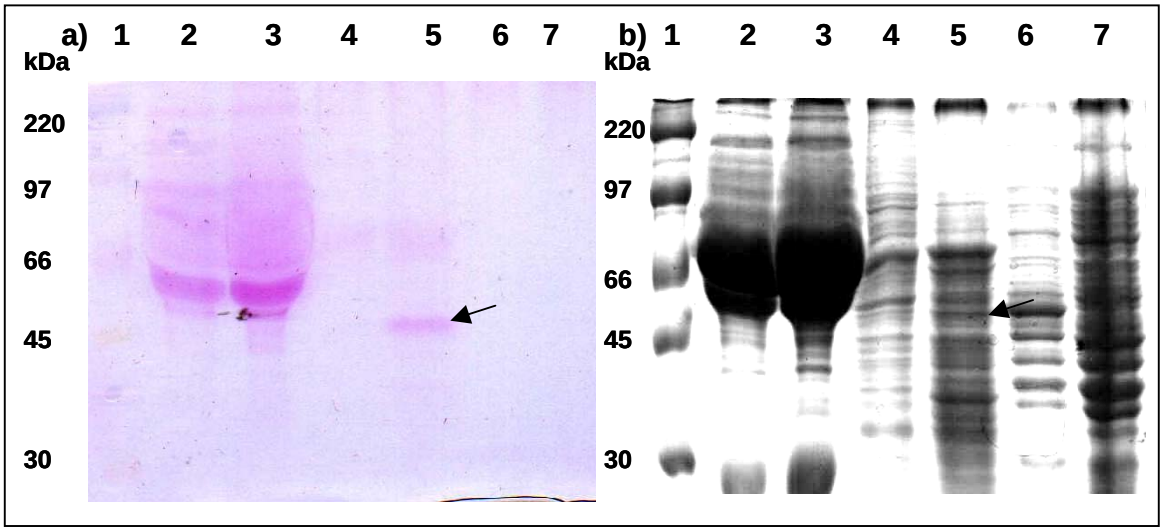

Figure 3.7 Glycosylation assay of VP6 of AHSV-6.

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

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

3.4 DISCUSSION

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

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

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

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

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

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

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

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

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

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

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