CHAPTER 2:

EXPRESSION OF AHSV-3 NS3 AND NS3A AS HISTIDINE TAG FUSION PROTEINS AND ANALYSIS OF THEIR CYTOTOXIC EFFECT

2.1 INTRODUCTION:

The AHSV nonstructural proteins, NS3 and NS3A, are encoded from two in-frame overlapping reading frames on segment 10 (Van Staden and Huismans 1991). The cognate genes in related orbiviruses also encode two almost identical proteins, which differ only with respect to an additional short N-terminal extension in the longer isoform. The reason for this conserved feature is not yet clear and was addressed in this study.

The segment 10 products may be related to other cytolytic proteins or viroporins, based on the finding that NS3 and NS3A are cytotoxic to insect cells, together with the presence of characteristic viroporin structural features in these proteins (Van Niekerk *et al.*, 2001a). The effect of NS3 and NS3A on mammalian cell membranes has not yet been investigated, and the proteins have not been expressed in a mammalian expression system. Several cytolytic proteins such as NSP4 of rotavirus (Tian *et al.,* 1996; Tafazoli *et al.,* 2001) and HIV-1 virus protein R (Vpr) (Macreadie *et al.,* 1996) have been shown to exert their membrane destabilising activity on prokaryotic and eukaryotic cells when expressed endogenously and when added, in purified form, exogenously to cell cultures. The effect of the exogenous addition of NS3 on the membrane permeability of mammalian cells was therefore investigated here.

Expression of the segment 10 gene from a recombinant baculovirus in insect cells results in the synthesis of NS3 only, at low levels (Van Staden *et al.,* 1995). Expression of NS3A was achieved by the removal of the nucleotides preceding the second initiation codon (Van Niekerk *et al.,* 2001a). Both proteins were found to be cytotoxic to insect cells when expressed individually, causing cell membrane permeability and eventual cell death (Van Staden *et al.,* 1995;

Van Niekerk *et al.,* 2001a). In this study the cytotoxic effect on insect cells of co-expression of NS3 and NS3A was investigated, as AHSV infected cells show expression of both proteins in equimolar amounts. This was achieved by co-infection of insect cells with recombinant baculoviruses expressing NS3 and NS3A, and monitoring cell death.

The availability of purified NS3 and NS3A would allow for further comparative and functional studies. The NS3 and NS3A proteins were, therefore, expressed as histidine fusion proteins in the baculovirus expression system.

The Bac-to-Bac™ baculovirus expression system provides a way of producing high levels of protein expression in an eukaryotic expression system. This has the advantage over prokaryotic systems in that the recombinant proteins are processed more correctly with respect to post-translational modifications such as disulphide bonds, glycosylation and phosphorylation and are therefore also likely to fold properly. It has been reported that many recombinant proteins expressed by means of the baculovirus expression system are functionally similar to their authentic counterparts (O'Reilly *et al.,* 1992; Luckow *et al.,* 1993). The production of recombinant baculoviruses expressing NS3 and NS3A as histidine tag fusion products would allow for the purification of the proteins and subsequent functional studies.

2.2 MATERIALS AND METHODS

2.2.1 Materials obtained:

Plasmids:
pAM25 (Dr V van Staden, Department of Genetics, University of Pretoria) AHSV-3 NS3 gene cloned into the *Bam*HI site of pUC13.
pBS-NS3H (AE Mercier, Department of Genetics, University of Pretoria) AHSV-3 NS3 gene cloned into *Bam*HI site of pBS, sequence not confirmed.
pBacHTc-NS3 (AE Mercier) AHSV-3 NS3 gene, subcloned from pBS-NS3H, into *Nar*I and *Bam*HI sites of baculovirus transfer vector pFastBacHTc, sequence not confirmed.

Recombinant baculoviruses:
Bac-NS3 (CC Smit, Department of Genetics, University of Pretoria) recombinant baculovirus expressing AHSV-3 NS3 from polyhedrin promoter.
Bac-NS3A (CC Smit) recombinant baculovirus expressing AHSV-3 NS3A (truncated S10 gene lacking first 30 nucleotides) from polyhedrin promoter.

Antisera:
α-β-gal-NS3: (Dr V van Staden) polyclonal antiserum raised in rabbits to a denatured form of a bacterially expressed β-galactosidase-NS3 fusion protein.
α-NS3: (Dr V van Staden) polyclonal antiserum raised in rabbits to a denatured form of AHSV-3 NS3 expressed in insect cells as a recombinant baculovirus protein.

Primer:
NS3PEco: (M van Niekerk, Department of Genetics, University of Pretoria) primer complementary to the 3' end of AHSV-2/3 S10 gene, for the amplification of NS3/A gene, that includes an *Eco*RI site for cloning purposes.

2.2.2 Exogenous addition of NS3 to Vero cells

2.2.2.1 Cells and viruses:

The GIBCO BRL BAC-to-BAC^{TM} baculovirus expression system was used for all protein expressions (Life Technologies, Luckow *et al.*,1993). *Spodoptera frugiperda* (*Sf9*) cells were used for baculovirus infections (American Type Culture Collection (ATCC), CRL1711, supplied by Sterilab). *Sf9* cells were maintained in confluent monolayers or as suspension cultures in spinner flasks at 27°C in Grace's insect medium containing 10% foetal calf serum (FCS), as well as antibiotics and fungizone (Highveld Biologicals). Cell culture techniques are essentially as described in the BAC-to-BAC^{TM} baculovirus expression system manual (GIBCO BRL, Life Technologies).

Cell density and viability were determined by the addition of the vital exclusion dye, Trypan Blue (0.4%), in a 1:1 ratio and inspection under a haemocytometer. Cells that were able to exclude the dye were considered viable. Typically spinner or shaker cultures were seeded at $0.2x10^6$ cells/ml and allowed to grow to a density of $2x10^6$ cells/ml in a final volume of 50-100 ml.

2.2.2.2 Preparation of *Sf9*-cell lysates containing NS3

Sf9 cell monolayers seeded at 1×10^7 cells in 75 cm^3 flasks were infected with wildtype baculovirus, or infected with a recombinant baculovirus expressing NS3 (Bac-NS3) at a Multiplicity of Infection (MOI) of 4 plaque forming units per cell (pfu/cell). The cells were harvested 30 hours post infection (h.p.i), resuspended in lysis buffer without detergent (50 mM Tris-HCl, pH 7.5; 300 mM NaCl; 1 mM PMSF, 1 mM 2-mercaptoethanol) at 2×10^7 cells/ml and mechanically lysed with a dounce homogeniser. Protein expression was

confirmed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot. Cell lysates were placed on ice and used immediately.

2.2.2.3 SDS-PAGE:

Protein expression from recombinant baculoviruses was analysed by electrophoresis under denaturing conditions in SDS-PAGE gels. As described by Sambrook *et al.* (1989) this method allows for the separation of proteins based on their molecular size. 12% or 15% separating (12% or 15% polyacrylamide; 0.375 M Tris-HCl, pH 8.8; 0.1% SDS; 0.008% TEMED; 0.08% ammonium persulphate) and 5% stacking (5% polyacrylamide; 0.125 M Tris-HCl, pH6.8; 0.1% SDS; 0.008% TEMED; 0.08% ammonium persulphate) polyacrylamide gels were prepared and cast between 7x10 cm glass plates. Protein samples were denatured in an equal volume of Protein Solvent Buffer (0.125 M Tris, pH 8; 4% SDS; 20% glycerol; 10% 2-mercaptoethanol) by heating at 96°C for 5 min. DNA was degraded by sonication for 10 min to decrease the viscosity of the sample. The denatured samples were then electrophoresed at 120-130 V in 1xTGS (0.3% Tris; 1.44% Glycine; 0.1% SDS) using the Hoefer Mighty Small^{TM} II SE 250 unit for 2 hours.

2.2.2.4 Western immunoblots:

Protein samples for immunoblotting were first separated by SDS-PAGE (2.2.2.3). Proteins were then transferred to Hybond-C membranes (AEC Amersham) in a submerged blotting apparatus (EC 140 miniblot module). After transfer was completed the gel was removed and stained in 0.125% Coomassie Blue, 50% methanol, 10% acetic acid and destained in 5% acetic acid, 5% methanol to verify transfer. The membrane was washed for 5 min in 1x PBS and non-specific binding sites blocked by incubation at room temperature for 30 min in 1% blocking solution (1% milk powder in 1x PBS). The membrane was then incubated overnight in a 1/100 dilution of the primary antibody, in this case a rabbit monospecific serum directed against a β-gal-NS3 fusion protein, in 1% blocking solution. The antiserum was then removed and the membrane washed three times for 5 min each in wash buffer (0.05% Tween in 1x PBS). A secondary antibody solution, a 1/1000 dilution of protein A conjugated to horseradish peroxidase (Cappel) in blocking solution, was then incubated with the membrane for 1 h. Unbound antibody was removed by washing three times in wash buffer and once in 1x PBS for 5 min each. Antibody binding was visualised by the addition of enzyme substrate (a mixture of 60mg 4-chloro-1-naphthol in 20ml methanol and 60µl hydrogen peroxide in 100ml 1xPBS). The reaction was allowed to proceed until bands became visible. Blots were rinsed with dH_2O and air-dried.

2.2.2.5 Preparation of Vero cells

Vero cells were maintained as confluent monolayers in 75cm^3 flasks at 37°C with 5% CO_2 in MEM (minimal essential medium) supplemented with 5% FCS and antibiotics (Highveld Biologicals).

For membrane permeabilisation assays, Vero cells were seeded on 24-well plates at 50% confluency and incubated overnight at 37°C with 5% CO_2 in MEM growth medium supplemented with 5% FCS and antibiotics (Highveld Biologicals). Twelve wells were seeded for each time interval to be assayed.

2.2.2.6 Hygromycin B membrane permeabilisation assay

Membrane permeabilisation assays were modified from the method described by Chang *et al.*,(1999). Growth medium was removed from Vero cells and replaced with equimolar amounts of crude cell extracts from *Sf9* cells infected with wild type baculovirus or Bac-NS3 prepared under 2.2.2.2 and supplemented with MEM growth medium. Six wells of Vero cells were treated in this way for each time interval for each *Sf9* cell lysate preparation. After 45, 90, 135 and 180 min the lysates were removed and MEM medium without methionine added with or without Hygromycin B (Hyg B;500µg/ml, Roche Diagnostics), each in triplicate for each of the wildtype baculovirus or Bac-NS3 infected *Sf9* cell lysate preparations. Cells were starved of methionine in the presence or absence of Hyg B for 30 min. The medium was replaced with MEM without methionine and 100 µCi ^{35}S-methionine (Separations) and labelling allowed to occur for 30 min. Vero cells were kept at 37°C with 5% CO_2 throughout the assay. The medium was removed and the cells rinsed twice in PBS to remove any remaining traces of ^{35}S-methionine. The cells were lysed in 50 µl lysis buffer (1% Nonidet P-40; 150 mM Tris-HCl pH 7.5; 1 mM EDTA with 20 µg/ml PMSF). A 50 µL TCA solution (5% trichloroacetic acid; 20 mM sodium pyrophosphate) was then added to this to precipitate proteins. A 50 µL sample of the precipitated proteins was blotted onto fibreglass discs (GF/C Whatman). The discs were washed with 70% ethanol, air-dried and placed in scintillation fluid (Beckman) and counted in a Beckman 3801 β counter. The percentage ^{35}S-methionine incorporated into permeabilised cells was calculated as follows:

$$\text{Percentage permeabilised VERO cells} = \frac{\text{Incorporation of } S^{35} \text{ in presence of Hyg B}}{\text{Total protein (Incorporation in absence of Hyg B)}} \times 100$$

Calculated values were used to plot ^{35}S-methionine incorporation graphs.

2.2.3 Co-expression of AHSV-3 NS3 and NS3A in insect cells:

2.2.3.1 Determination of cell viability by trypan blue staining

50 ml spinner cultures of *Sf9* cells at 1 x 10^6 cells/ml were infected with baculoviruses at a MOI of 5-10 pfu/cell. 100 µl aliquots of infected cells were removed every 3 hours from 0-48 h.p.i and stained in an equal volume of 0.4% trypan blue in 1x PBS. The number of stained cells were counted using a haemocytometer and expressed as a percentage of the total cell population. Protein expression was confirmed by SDS-PAGE analysis and Western blot.

2.2.4 Cloning of the AHSV-3 NS3A gene into pFastBacHTc:

2.2.4.1 Polymerase chain reaction:

Two primers were designed to specifically amplify the region of the AHSV-3 S10 gene encoding NS3A. The primer sequences were 5'-CGTTCGAA ATGATGCATAATGAATC-3' (NS3Ahfor) and 5'-CG***GAATTC***GTAAGTCGTTA TCCCGG-3'(NS3pEco). The sequences incorporated a *Sfu* I site (underlined) at the 5' end and an *Eco*RI site (bold italic) at the 3' end of the gene for insertion of the NS3A gene into the pBS and pFASTBAC HTc vectors. PCR reactions were set up as follows: 50 ng pAM25, 50 μmol of each dNTP, 1.5 mM $MgCl_2$, 10 μl 10 x reaction buffer (Promega) and 100 pmol of each primer were mixed and made up to 99.5 μl with UHQ. The DNA template was denatured at 95°C for 5 min in a thermocycler ("hot start" PCR) and 0.5 μl *Taq* DNA polymerase (5U/μL, Promega) added. A Hybaid (Omnigene) programmable thermocycler was used to perform 30 cycles of PCR. The primary denaturation was followed by an annealing cycle of 45 sec at 53°C as determined by the primer sequence. Primer extension occurred at 72°C for 45 sec. This primary cycle was followed by 28 cycles where denaturation took place at 95°C, primer annealing at 55°C for 45 sec and extension at 72°C for 3 min. The final cycle allowed for elongation for 5 min. PCR products were then analysed by 2% agarose gel electrophoresis in 1XTAE (0.04 M Tris-acetate, 1 mM EDTA, pH 8.5) using a Biorad Mini Sub™ electrophoresis unit. The addition of 1% Ethidium Bromide to the agarose gels allowed for the visualisation of the DNA under UV light. The size of the DNA fragments was estimated by comparison of their migration during electrophoresis with standard molecular weight size markers (*Hae*III digested ϕX174, Promega and Lambda *Hin*dIII digested DNA (SMII), Roche).

2.2.4.2 Plasmid DNA isolation:

Single bacterial colonies were selected from LB-agar plates. These colonies were used to inoculate 3ml liquid cultures in Luria-Bertani (LB) medium (1% bacto-tryptone (m/v), 0.5% bacto-Yeast extract (m/v), 1% NaCl (m/v), pH 7.4) in the presence of ampicillin (100 μg/ml) and/or tetracyclin (12.5 μg/ml). Cultures were amplified at 37°C overnight.

Plasmid isolations were done according to the alkaline-lysis method of Birnboim and Doly (1979) as described by Sambrook *et al.* (1989). Briefly, the overnight cultures were harvested by bench-top centrifugation for 1 min, and the pellet suspended in 100 μL of ice-cold lysis buffer containing 25 mM Tris-HCl, pH 8.0, 50 mM glucose and 10 mM EDTA. This mixture was incubated at room temperature for 5 min and on ice for 1 min. 200 μl 0.2 N NaOH, 1% SDS (alkaline-SDS buffer) was added to lyse the resultant spheroplasts, and incubated on ice for a further 5 min. Precipitation of protein, high molecular weight RNA and bacterial genomic DNA with 150μl ice-cold 3 M NaAc pH 4.8 for 5 min on ice, allowed for the removal of these contaminants. Plasmid DNA was then separated from the SDS-precipitated contaminants by centrifugation for 10 min. Plasmid DNA in the recovered supernatant was precipitated by the addition of 2 volumes of 96% ethanol at –20°C for 30 min. The precipitate was collected

by desktop centrifugation, washed with 80% ethanol, dried under vacuum, and resuspended in 30 µl 1XTE buffer (10 mM Tris, pH7.4, 1 mM EDTA, pH 8). Isolated plasmid preparations were analysed by 1% agarose gel electrophoresis (2.2.4.1).

2.2.4.3 Phenol-chloroform extraction:

Deproteination of DNA was achieved by phenol-chloroform extractions. An equal volume of a 1:1 phenol and chloroform mixture was added to the DNA samples to denature any proteins present. The denatured proteins in the phenol were separated from the DNA by desktop centrifugation, and the upper aqueous layer containing the DNA removed. 1 volume of chloroform was added to the DNA to remove any residual phenol. Precipitation of the DNA was carried out in 2 volumes 96% ethanol in the presence of 1/10 volume 3M NaAc. After centrifugation for 30 min the DNA was washed with 80% ethanol, vacuum-dried and resuspended in TE buffer (Ausubel *et al.*,1988).

2.2.4.4 Optical density analytical measurements of plasmid DNA concentration:

The concentration of DNA in purified samples was determined by measurement of the optical density at 260 nm (OD_{260}). The DNA sample was diluted 100x in Ultra High Quality H_2O (UHQ) and the OD_{260} determined using pure UHQ H_2O as the calibration medium. The concentration of the DNA was calculated using the extinction coefficient for double stranded DNA, $1A_{260} \equiv 50$ µg/ml and multiplication with the dilution factor. The purity of the sample was estimated by measuring the OD_{280} and determining the ratio of OD_{260} to OD_{280}. A ratio above 1.6 was taken as indicative of protein contamination and the DNA purified further using the phenol-chloroform extraction method (2.2.4.3). The presence of residual salt was evaluated by determining the OD_{320}. Samples with a reading higher than 0.001 were re-precipitated and washed in 70% ethanol to ensure the removal of the salt.

2.2.4.5 Klenow enzyme fill-in reactions:

Klenow enzyme was used to fill in overhangs to create blunt ends. Approximately 1 µg of DNA from PCR reactions was mixed with 2 µl Klenow enzyme(2 U/µl), 3 µl 10x buffer B (10 mM Tris HCl, 5 mM MgCl, 1 mM 2-mercaptoethanol)(Boehringer Mannheim), 2 µl dNTPs (5 mM of each dNTP) and made up to 30 µl with ddH_2O. The reaction was performed at 37°C for 30 min after which the enzyme was heat inactivated at 60°C for 10 min and the DNA allowed to reanneal at room temperature for 10 min.

2.2.4.6 Restriction enzyme digestions:

All restriction enzyme reactions were carried out in the restriction enzyme's recommended salt buffer, at its optimal pH and temperature (Promega or Boehringer Mannheim). In general reactions were performed in a final volume of 20µl for 1-2 hours. Digested DNA products were analysed on 1% agarose

gels. The size of the DNA fragments was estimated by comparison of their migration during electrophoresis with standard molecular weight size markers (*Hae*III-digested ϕX174, Promega and *Hin*dIII-digested Lambda DNA (SMII), Roche).

2.2.4.7 Purification of DNA fragments from agarose:

Restriction fragments to be isolated were excised from 1% agarose gels with a razor and purified using the Geneclean™ II reaction Kit (Bio101). In this procedure a silica matrix (glassmilk) is used, that binds selectively and specifically to ds- and ssDNA. The excised gel was melted at 55°C in 2.5 volumes 6M NaI. The DNA was then allowed to bind to 5 µl glassmilk at room temperature for 30 min, followed by 5 min on ice. DNA bound to the glassmilk was then collected by desktop centrifugation and washed 3x with 500 µl NEW Wash solution (NaCl, EDTA, EtOH and H_2O). The washed DNA was then eluted in TE buffer or UHQ H_2O at 45-55°C.

2.2.4.8 Ligation of DNA fragments:

Purified restriction fragments were ligated to the linearised vector using 1µL of a 10x ligation buffer (66 mM Tris-HCl, pH 7.5; 5 mM $MgCl_2$; 1 mM ATP) and 1 unit of T4 DNA Ligase in a reaction volume of 10 µL (Boehringer Mannheim). Generally a ratio of 3:1 of insert DNA to vector was used. The reaction was carried out at 16°C for 16 hours.

2.2.4.9 Preparation of competent cells:

The $CaCl_2$ method described by Sambrook *et al* (1989) was used to prepare competent XL1-Blue (*E. coli*) cells. 1 ml of an overnight culture was used to inoculate 100 ml LB-broth, and the cells allowed to grow to log phase (OD_{550} of 0.5) by shaking at 37°C. Cells were then collected by centrifugation at 5000 rpm for 5 min, and resuspended in half the original volume of ice-cold 50 mM $CaCl_2$. After incubation on ice for 30 min, the cells were again collected by centrifugation and resuspended in 1/20 of the original volume in $CaCl_2$. Competent cells were snap-frozen in liquid Nitrogen and stored at -70°C in glycerol or placed on ice for 1 h prior to use.

2.2.4.10 Transformation of recombinant DNA into competent cells:

100 µl of competent cells were mixed with 5 µl of ligation mixture and placed on ice for 30 min to allow for the binding of the DNA to the cell membranes. Transformation of the DNA into the cells was made possibly by a brief heat shock for 90 seconds at 42°C, followed by 2 min on ice. 1 ml pre-warmed LB-broth was then added and the culture incubated for 1 h at 37°C, to allow for the expression of the antibiotic resistance genes. 150 µl samples of the cultures were plated onto LB-agar plates (1.2% agar in LB medium) containing 100 µg/ml ampicillin and 12.5 µg/ml tetracyclin. 50 µl 2% X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and 10 µl 100mM/2% IPTG (isopropyl-β-D-thiogalactopyranoside) was added to plates streaked with XL1-Blue cells

transformed with pBS recombinants for blue-white selection. Plates were incubated at 37°C overnight. Recombinants that were successfully transformed with pBS with the insert were identified as white colonies that grew in the presence of the appropriate antibiotics. For XL1-Blues transformed with pFastBac recombinants selection was performed by restriction digestion of plasmid DNA.

2.2.5 DNA sequencing:

2.2.5.1 Plasmid purification for cycle sequencing

Plasmid DNA used for sequencing was purified using one of the two commercially available kits for the isolation of high quality DNA (Quigen plasmid mini purification protocol; Boehringer Mannheim High Pure Plasmid Isolation Kit Protocol). The kits were used according to the instructions supplied by the manufacturer, the principle being essentially the same as the alkaline-lysis method described above. In short, the cells were grown as before but lysed in an RNase-containing lysis buffer (50 mM Tris-HCl, pH 8.0; 1.0 mM EDTA; 100 µg/ml RNase A). Purification columns containing either anion exchange resin under the appropriate pH and low salt conditions (750 mM NaCl; 50 mM MOPS, pH 7; 15% isopropanol; 0.15% Triton X-100, Qiagen) or a "high pure filter-tube" where nucleic acids are specifically bound to the surface of glass fibres or silica materials in the presence of a chaotropic salt (Boehringer Mannheim) were used to bind the plasmid DNA. Unbound traces of proteins, RNA, dyes and low molecular weight impurities were removed by washing the column with a medium salt buffer (1.0 M NaCl; 50 mM Tris-HCl, pH 8.5; 15% isopropanol). Elution of the plasmid DNA was then achieved either by direct elution in UHQ H_2O (Boehringer Mannheim) or by a high salt buffer treatment (1.25 M NaCl; 50 mM Tris-HCl, pH 8.5; 15% isopropanol) followed by precipitation to remove salt in 0.7 volumes isopropanol at room temperature, washing in 70% ethanol, freeze-drying and resuspension in UHQ H_2O (Quigen).

2.2.5.2 Automated sequencing

DNA sequencing was performed by cyclic enzymatic extension reactions using dye-labelled terminators. The sequence reactions were analysed using the ABI Prism™ 310 Genetic Analyser (Perkin-Elmer). The automated cycle sequencing method was adapted from the Sanger method of sequencing (Sanger *et al.,* 1977; Prober *et al.,* 1987).

Briefly, 300 ng purified plasmid DNA was mixed with 8.0 µL Terminator ready reaction mix (A/C/G/T-dye terminators, dITP, dATP, dCTP, dTTP, Tris-HCl (pH 9.0), $MgCl_2$, thermal stable pyrophosphatase, and AmpliTaq DNA polymerase FS). 3.2-5 pmol M13 specific forward or reverse primers were used to sequence recombinant pBS subclones, as pBS contains the same MCS as M13 vectors (Stratagene). The final volume was made up with ddH_20 to 20 µl. The MJ-PTC-200 Thermal cycler was used for the cycle sequencing reactions (Peltier thermal cycler) using thin wall 500 µl eppendorf tubes. Thermal cycling

was performed according to the protocol specified by the Perkin Elmer thermal cycler.

Thermal cycling was started with a rapid thermal ramp to 96°C followed by 30 seconds at 96°C to denature the template. This was followed by a rapid ramp to 50°C. Elongation was accomplished by a rapid ramp to 60°C followed by 4 min at this temperature. This cycle was repeated for 25 cycles. After which a ramp to 4°C followed where the reaction was stopped and held until removed from the Thermal cycler.

The cycle sequencing reaction was precipitated by either NaAc or ammonium acetate (NH_4Ac) in 100% EtOH and vacuum dried. The dried sequencing extension products were resuspended in template suppression reagent (Perkin Elmer), heated to 95°C for two min to denature the DNA and placed on ice until ready for use. Sequencing reactions were then run on the ABI PrismTM 310 for analysis. The Sequencing Analysis and Sequence Navigator (Perkin Elmer) were used to analyse raw data produced and compare multiple sequences of different sequencing reactions of the same sequence as described in the ABI Prism Comparative PCR sequencing guide, Perkin Elmer (1995).

2.2.6 Baculovirus expression of histidine tagged NS3 and NS3A:

2.2.6.1 Preparation of competent DH-10Bac cells:

Competent DH10Bac™ cells were prepared according to the DMSO method of Chung and Miller (1988). A 3 ml overnight culture of DH-10Bac cells was used to inoculate 100 ml LB medium and allowed to grow to early log phase at 37°C. Cells were then collected by centrifugation at 5000 rpm for 5 min at 4°C. The pelleted cells were resuspended in 1/10 of the original volume of ice-cold TSB (1.6% Peptone; 1% yeast extract; 0.5% NaCl; 10% polyethyleneglycol; 1 M $MgCl_2$; 1 M $MgSO_4$) and incubated for 20 min on ice before transformation.

2.2.6.2 Transposition of recombinant genes into the bacmid DNA:

100 ng of each of the donor plasmids, pFastBac-HTc containing the NS3 and NS3A genes, was mixed with 100 µl competent DH10Bac™ cells and incubated on ice for 20 min. A heat shock for 45 sec at 42°C allowed for the transformation of the DNA into the cells. 900 µl TSBG growth medium (TSB with 20 mM glucose) was added to each transformation mixture and the cells incubated at 37°C for 4 hours. 100 µl of each culture was plated onto LB-agar plates containing 50 µg/ml kanamycin sulphate, 12.5 µg/ml gentamycin, 40 µg/ml IPTG and 50 µL of 2% X-gal per agar plate. The plates were incubated at 37°C for 24 hours. White colonies were selected and plated onto the same medium to ensure that they were recombinant composite Bacmid containing cells.

2.2.6.3 Isolation of composite bacmid DNA:

Recombinant colonies identified in 2.2.6.2 were used to inoculate 2 ml LB-broth containing 50 µg/ml kanamycin and 7 µg/ml gentamycin and incubated at 37°C for 16 h with shaking. The cells from 1 ml of these cultures were harvested

by desktop centrifugation for 1 min. The protocol adapted from Amemiya *et al.* (1994) for the isolation of high molecular weight Bacmid DNA was used (BAC-to-BAC™ Baculovirus expression system manual). In short, the DH10BAC cell pellet was resuspended in 300 µl of solution 1 (25 mM Tris-HCl, pH 8; 50 mM glucose and 10 mM EDTA) for disruption of the cell membranes. The addition of 300 µl of an alkaline-SDS buffer (0.2 N NaOH; 1% SDS) for 5 min at room temperature allowed for the denaturation of proteins and DNA. The pH of the solution was decreased through the addition of 300 µl solution 3 (3 M Potassium acetate, pH 5.5), so allowing for the precipitation of proteins and genomic DNA and the reannealing of the plasmid DNA. After incubation on ice for 10 min, the insoluble material was removed from the solution by centrifugation at maximum speed for 10 min. The plasmid DNA in the supernatant was precipitated in 800 µl isopropanol on ice for 10 min. The DNA was collected by desktop centrifugation for 15 min at maximum speed. The recombinant Bacmid DNA was then washed with 70% ethanol, air-dried briefly and resuspended in 40 µl TE buffer.

2.2.6.4 Transfection of *Sf9* cells with bacmid DNA:

Six well (35mm) tissue culture plates were seeded with $1x10^6$ cells/well in 2 ml Grace's medium with antibiotics (Highveld Biologicals) and allowed to attach for 1 h. Recombinant Bacmid DNA isolated in 2.2.6.3 was transfected into insect cells by lipofection (Felgner *et al.*,1987). For each transfection reaction 6 µl of Bacmid DNA was diluted in 100 µl Grace's medium without antibiotics. 6 µl CELLFECTIN™(BRL) reagent was mixed with 100 µl Grace's medium without antibiotics in a separate eppendorf. The diluted Bacmid DNA and CELLFECTIN™ were then mixed and incubated at room temperature for 30 min. 0.8 ml Grace's medium without antibiotics or serum was then added to the lipid DNA complexes. The attached cells were washed twice with Grace's medium without serum or antibiotics and overlayed with the lipid DNA complexes. The cells and DNA were incubated together at 27°C for 5 h after which the DNA was removed and replaced with 2 ml Grace's medium containing antibiotics and serum. The transfected cells were incubated for a further 96 h at 27°C. Recombinant Baculoviruses in the medium were removed and stored at 4°C for later use.

2.2.6.5 Amplification of baculovirus recombinants:

Virus stocks were produced by infecting monolayers of $1x10^7$ cells seeded in 75 cm^3 flasks at a low MOI of 0.1 pfu/cell. Amplification was carried out for 72 h at 27°C after which the medium containing the virus was clarified by centrifugation at 2000 rpm for 5 min and filter sterilisation. Virus stocks were stored at 4°C or at -70°C for long term storage.

2.2.6.6 Titration of viruses:

Sf9 cells were seeded in 35mm 6-well plates at a density of 1.5×10^6 cells/well and allowed to attach for 1 h. Dilution series of the viruses to be titrated were prepared, from 10^{-1} to 10^{-9} in 1 ml Grace's medium. The medium from each

well was removed and replaced with the virus dilutions. The plates were incubated at room temperature for 2 h, after which the inoculum was replaced with sterile 3% low melting agarose (Gibco BRL) at 37°C diluted in an equal volume Grace's medium. The infected cells were incubated at 27°C for 4 days and then stained overnight or for 3-5 hours with 1 ml Neutral Red (100 µg/ml in Grace's medium). Viral plaques appeared as light red patches in a darker red background.

2.2.6.7 Analysis of protein expression:

Sf9 cells were infected with recombinant baculoviruses in 6 well plates seeded at $1x10^6$ cells/well at a MOI of 5-10 pfu/cell. The cells were harvested after 72 h incubation at 27°C for the analysis of protein expression. Cells were collected by centrifugation at 3000 rpm for 5 min and resuspended in 1x PBS. The infected cells were then washed again in PBS and analysed immediately by SDS-PAGE (2.2.2.3) and Western blot (2.2.2.4) or stored at -20°C.

2.2.7 Protein solubility analyses:

2.2.7.1 Basic solubility assays:

Sf9 cells were seeded at a density of 1×10^7 cells in 75 cm^3 flasks and infected with recombinant baculoviruses at a MOI of 2-5 pfu/cell. Cells were harvested 48 – 72 h.p.i by centrifugation at 3000 rpm for 5 min. Cells were then resuspended in lysis buffer A [50 mM Tris-HCl (pH 7.5), 1 mM 2-mercaptoethanol and 1 mM PMSF (phenylmethylsulfonyl fluoride)] at 5 volumes lysis buffer/g cells and incubated on ice for 30 min. In order to investigate the solubility of the histidine tagged protein, in different detergents at different concentrations, this standard lysis buffer was adjusted to contain 0.1%, 0.25%, 0.5%, 1.0% or 2.0% Nonidet P-40 or Triton X-100 respectively. The cells were then mechanically lysed using a needle with a gauge of 22 (Promex) or disrupted with a dounce homogeniser. Insoluble proteins were collected by centrifugation at 2000g for 10 min and resuspended in an equal volume to the soluble proteins in the supernatant. Proteins were analysed by 12% SDS-PAGE (2.2.3.3) and Western blot (2.2.3.4). To determine the solubility of the HTc-NS3 protein in the presence of various salt concentrations (0.15 M, 0.3 M and 0.5 M NaCl) the solubility assay was repeated as above with lysis buffer A containing 1.0% Triton X-100 and the indicated salt concentrations.

2.2.7.2 Binding of HTc-NS3 to Ni^{2+}- resin

1×10^7 *Sf9* cells in 75 cm^3 flasks were infected with BacHTc-NS3, harvested 48 h.p.i. and resuspended in lysis buffer (lysis buffer A + 1.0% Triton X-100, 0.3 M NaCl). Cells were lysed as described in 2.2.7.1 and separated into soluble and insoluble fractions by centrifugation. Charged nickel resin (50, 100 or 200 µl, Gibco BRL, Life Technologies) was added to soluble proteins in the supernatant and allowed to bind for 30 min at 4°C. Samples were centrifuged at a low speed of 1000 rpm for 5 min to collect the resin and bound proteins. The unbound proteins in the supernatant were removed and retained. The resin was

then resuspended in and washed with wash buffer (20 mM Tris-HCl (pH 8.5); 500 mM KCl, 20 mM or 10 mM imidazole, 10 mM 2-mercaptoethanol, 10% (v/v) glycerol). The resin was collected by low speed centrifugation and the supernatant retained for later analysis. Bound proteins were then eluted through the addition of elution buffer (20 mM Tris-HCl (pH 8.5), 100 mM KCl, 100 mM imidazole, 10 mM 2-mercaptoethanol, 10% (v/v) glycerol) to the resin. Eluted proteins were separated from the resin by centrifugation at 1000 rpm for 5 min and retained for analysis. The elution was repeated to ensure that all bound proteins were removed. Samples collected at each stage during the assay were analysed by 12% SDS-PAGE and Western blot.

2.2.7.3 Comparison of solubility of HTc-NS3 and NS3:

In order to compare the solubility of the NS3 fusion protein (HTc-NS3) to the wild type NS3 protein, recombinant baculovirus infected cells were resuspended and lysed in lysis buffer A without detergent and separated into soluble and insoluble fractions as described above (2.2.7.1). Pellet fractions were then resuspended in lysis buffer A with 1.0% Triton X-100, 0.3 M NaCl and incubated on ice for 15 min. Samples were then fractionated again, to determine if the addition of detergent solubilised the protein. Alternatively infected cells were resuspended directly in lysis buffer A with 1.0% Triton X-100, 0.3 M NaCl, lysed and separated into soluble and insoluble fractions as before.

2.2.8 Sequence analysis of N-terminal residues of NS3 and NS3A

The N-terminal residues of NS3 and NS3A were analysed using the subcellular localisation site predictor programs, PSORT (http://psort.nibb.ac.jp/) and iPSORT (http://hypothesiscreator.net/iPSORT/).

PSORT is a computer program used to predict the subcellular localisation sites of proteins from their amino acid sequences using the *k*-nearest neighbours classifier (Horton and Nakai, 1996; Horton and Nakai, 1997). A subprogram within PSORT allows for the recognition or prediction of a signal sequence or leader peptide using McGeoch's method (McGeoch, 1985) with modifications. It considers the N-terminal positively-charged region (N-region) and the central hydrophobic region (H-region) of signal sequences. A discriminant score (termed the PSG score) is calculated from the three values: length of H-region, peak value of H-region and net charge of N-region. A large positive discriminant score means a high possibility to possess a signal sequence but is unrelated to the possibility of its cleavage. Next, PSORT applies von Heijne's method of signal sequence recognition (Horton & Nakai 1997). This is a weight-matrix method and incorporates the information of consensus pattern around the cleavage sites as well as the features of the H-region. Thus it can be used to detect signal-anchor sequences. A large positive output score (GvH score) means a high possibility that it has a cleavable signal sequence. The position of a possible cleavage site is also reported. Other PSORT subprograms allow for the prediction of

transmembrane segments and membrane topology amongst various other applications.

iPSORT is a subcellular localisation site predictor for N-terminal sorting signals. Given a protein sequence (maximum of 30 amino acids), it will predict whether it contains a signal peptide, using an alternative method to the ones described above.

The NS3 N-terminal residues were also scanned against protein profile databases (including Prosite) (http://isrec.isb-sib.ch/). The sequence was furthermore analysed using PattinProt (http://npsa-pbil.ibcp.fr), which scans a protein sequence or a protein database for one or several patterns. The consensus secondary structure of this region was also determined (http://npsa-pbil.ibcp.fr).

2.3 RESULTS

This part of the study aimed to further analyse the cytotoxic properties of AHSV NS3 and NS3A, and to compare these almost identical proteins. The effect of the exogenous addition of the NS3 protein to mammalian cells was investigated. The cytotoxicity of the endogenous proteins to insect cells when expressed either individually or together was also investigated and compared. To prepare large quantities of purified NS3 and NS3A, the NS3 and NS3A genes were cloned into the baculovirus histidine tag transfer vector pFASTBAC HTc. Expression of NS3 and NS3A as histidine fusion proteins would allow for their large scale purification on colomns, in a biologically active form for further functional analyses. The N-terminal region of the NS3 protein was also analysed computationally for the presence of a possible functional domain.

2.3.1 The effect of extracellular addition of NS3 on Vero cells

AHSV NS3 and NS3A have been shown to be cytotoxic to *Sf9* cells, causing membrane permeabilisation and eventual cell death (Van Staden *et al.*, 1995; Van Niekerk *et al.*, 2001a). BTV NS3, however, has not been reported to be cytotoxic when expressed in insect cells. The AHSV NS3 protein may be functionally related to the NSP4 protein of rotavirus (a member of the reoviridae family) discussed in section 1.3.2. The cytotoxic properties of NS3, however, require further investigation. One aspect of NS3 functioning that has not been addressed in the literature is whether the extracellular addition of AHSV NS3 to

cells has an effect on membrane permeability. The aim here was to investigate the effect, if any, of NS3 on the membranes of mammalian cells following extracellular addition of the protein.

As the NS3 protein has not yet been purified in a non-denatured form, it was decided to use crude cell extracts from *Sf9* cells infected with a recombinant baculovirus expressing NS3, for initial membrane permeabilisation assays. As a negative control, *Sf9* cell extracts from wild-type baculovirus infected cells were also prepared as described under Materials and Methods. Expression of the NS3 protein was confirmed by immunoblot with α-β-gal NS3 serum (Figure 2.1(A)). Extracts from Bac-NS3 infected *Sf9* cells contain a protein or band of approximately 24 kDa, that reacts positively with the α-β-gal-NS3 serum, this represents AHSV-3 NS3 (Figure 2.1(A), lane a). Wild type baculovirus infected cells do not have the 24 kDa band (Figure 2.1(A), lane b). Both lanes show a non-specific band at a position just below the 66 kDa size marker.

Approximately equimolar amounts of cell extracts were added to cultured Vero cells and the permeabilisation of the membrane monitored with Hyg B, at 45 min intervals over a 3h period. Hyg B is an aminoglycosidase that is effective in preventing the translocation step in cell-free translation systems. However Hyg B is a very ineffective inhibitor of protein synthesis in intact cells, as it does not cross the plasma membrane (Benedetto *et al.,* 1980). Only permeabilised cells therefore allow the entry of Hyg B, which then subsequently halts protein synthesis. S^{35} metabolic labelling of cellular proteins was used here to monitor protein synthesis in Vero cells, and thereby the uptake of Hyg B and membrane permeabilisation.

Figure 2.1(B) summarises the results from 9 repetitions of the experiment. Wildtype baculovirus-infected *Sf9* cell lysate caused only a slight increase in membrane permeabilisation of Vero cells, with 15% (± 2.6) permeabilised after 3 hours. The addition of *Sf9* cell lysates containing NS3 to Vero cells caused a much larger increase in membrane permeability, with 62% (± 3.8) permeabilisation after 3 h. There was therefore on average a 47% increase in the permeabilisation affected between the wild-type baculovirus- and NS3-

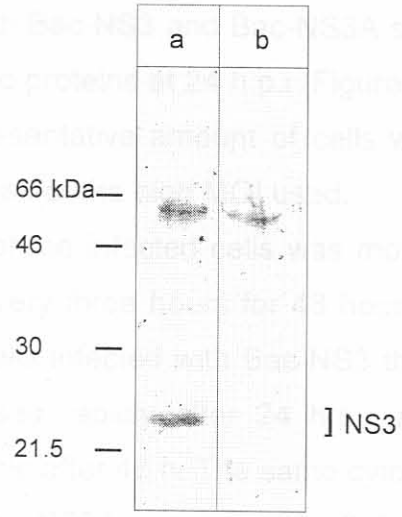
containing lysates. As the only difference between these lysates is the presence of the NS3 protein it is safe to assume that this difference is due to the presence of this protein. The NS3 protein, from this initial study, appears therefore to cause membrane permeabilisation in mammalian cells when added exogenously. Further investigations into this property of NS3 would be greatly aided by the availability of purified NS3, for this purpose the NS3 and NS3A proteins were expressed as histidine fusion products in the Baculovirus system (2.3.3)

2.3.2 Analysis of the cytotoxic effect of co-expression of NS3 and NS3A in insect cells

Both NS3 and NS3A are expressed together in AHSV infected cells (Van Staden *et al.,* 1995). Expression of the AHSV-3 S10 gene in insect cells, however, results in the synthesis of NS3 alone (Van Staden *et al.,* 1995). Expression of AHSV-3 NS3A in insect cells was achieved by the removal of the first initiation codon and bases preceding the second initiation codon. The NS3A protein was found, like NS3, to be cytotoxic to insect cells (Van Niekerk *et al.,* 2001a). It has, however, never been investigated if the combined expression of NS3 and NS3A has an enhanced cytotoxic effect over and above individually expressed NS3 and NS3A. Co-expression of the proteins could conceivably lead to interactions between the proteins that may affect their cytotoxicity. It was, therefore, decided here to investigate the effect on insect cells of the co-expression of these two proteins.

Sf9 cells were co-infected with Bac-NS3 and Bac-NS3A or infected with these baculoviruses separately. As a control, cells were infected with a recombinant baculovirus expressing AHSV NS2 (Bac-NS2) as this protein has been shown not to be cytotoxic to *Sf9* cells (Van Staden *et al.,* 1995). The expression of NS3 and NS3A was analysed at 18, 24, 36 and 42 h.p.i by immunoblot with the α-β-gal-NS3 serum, the results at 24 h.p.i are shown in Figure 2.2(A). Cells infected with Bac-NS3 or Bac-NS3A showed expression of NS3 (24K) or NS3A (23K) at 24 h.p.i (figure 2.2(A), lanes b and c respectively).

(A)



(B)

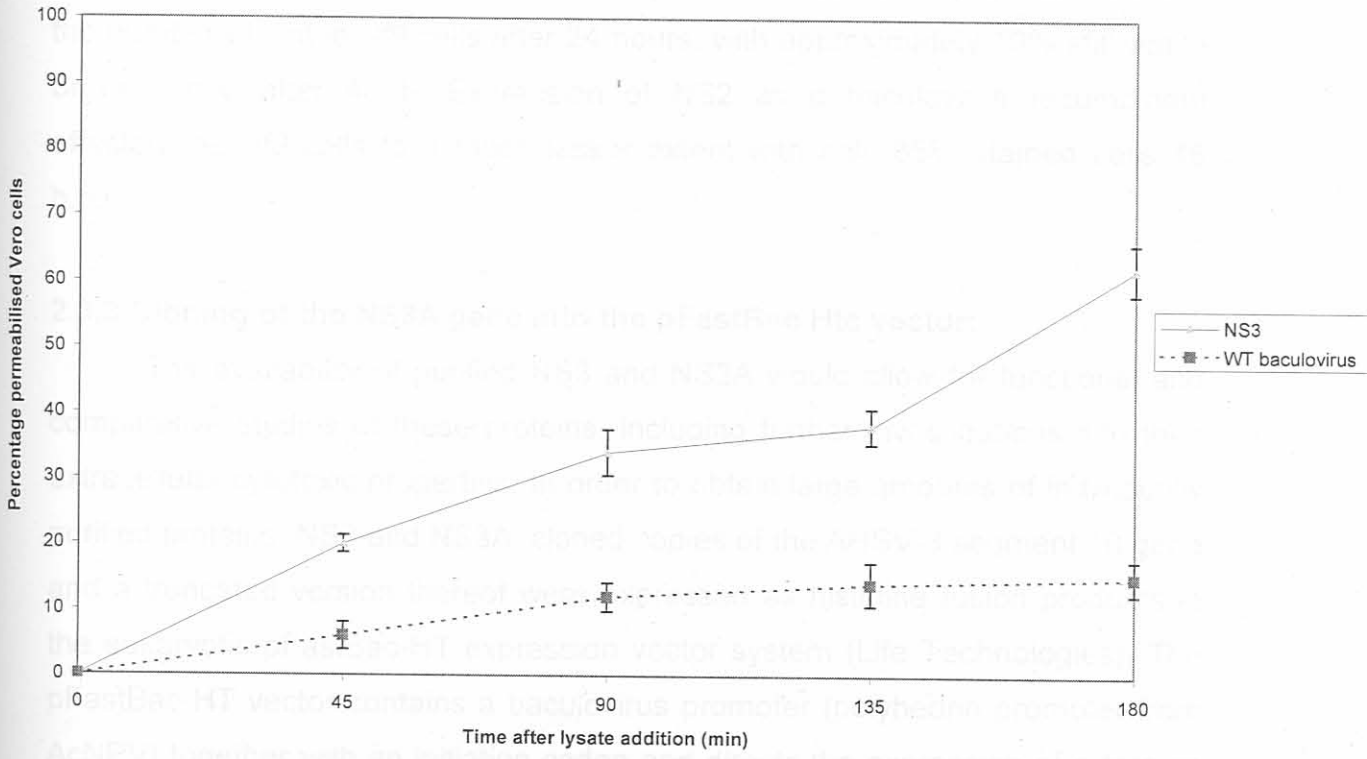


Figure 2.1(A) Western immunoblot analysis of expression of AHSV-3 NS3. *Sf*9 cells were infected with Bac-NS3 (a) or wild type baculovirus (b) and cell lysates prepared 30 h.p.i. Proteins were separated by 12% SDS-PAGE, blotted onto a nitrocellulose membrane and reacted with α-β-gal-NS3 serum. The molecular weights of the rainbow markers and the position of NS3 are indicated. **(B)** Hygromycin B assay of changes in Vero cell membrane permeability following exogenous addition of *Sf*9 cell lysates with or without NS3.

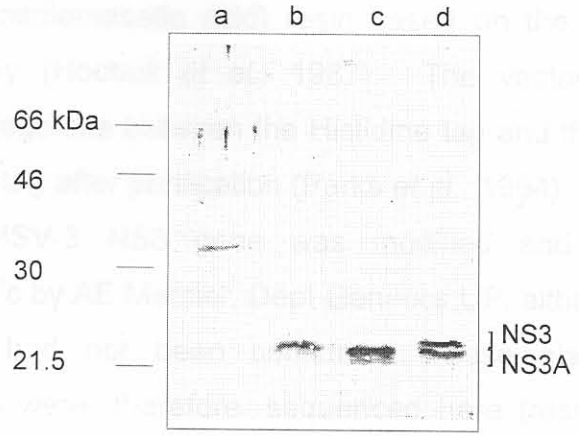
Cells co-infected with Bac-NS3 and Bac-NS3A showed approximately equimolar expression of the two proteins at 24 h.p.i (Figure 2.2(A), lane d). It was assumed that at least a representative amount of cells were co-infected and expressed both proteins, because of the high MOI used.

The viability of the infected cells was monitored using the vital exclusion dye, Trypan blue, every three hours for 48 hours. The results are illustrated in Figure 2.2. In *Sf9* cells infected with Bac-NS3 the number of cells unstained by trypan blue decreased rapidly after 24 h.p.i and only 7% of the cells were unstained or still viable after 48 h. The same cytotoxic effect was observed in *Sf9* cells infected with Bac-NS3A, with less than 20% of the cell population still viable after 48 h. These observations confirm the results from previous studies done by Van Staden *et al.* (1995) and Van Niekerk and co-workers (2001a). Cells co-infected with Bac-NS3 and Bac-NS3A displayed the same dramatic decrease in the number of viable *Sf9* cells after 24 hours, with approximately 10% still viable or unstained after 48 h. Expression of NS2 as a baculovirus recombinant affected the *Sf9* cells to a much lesser extent with only 35% stained cells 48 h.p.i.

2.3.3 Cloning of the NS3A gene into the pFastBac Htc vector:

The availability of purified NS3 and NS3A would allow for functional and comparative studies of these proteins, including further investigations into their extracellular cytotoxic properties. In order to obtain large amounts of individually purified proteins, NS3 and NS3A, cloned copies of the AHSV-3 segment 10 gene and a truncated version thereof were expressed as histidine fusion products in the eukaryotic pFastBac-HT expression vector system (Life Technologies). The pFastBac-HT vector contains a baculovirus promoter (polyhedrin promoter from AcNPV) together with an initiation codon and directs the expression of a foreign protein as a fusion protein with six histidine residues at its amino terminus. Three pFastBac-HT vectors are available (a, b and c) with unique restriction enzyme sites in different reading frames through the cloning linker. These histidine fusion proteins can be easily purified because of the strong affinity of the His-tag

(A)



(B)

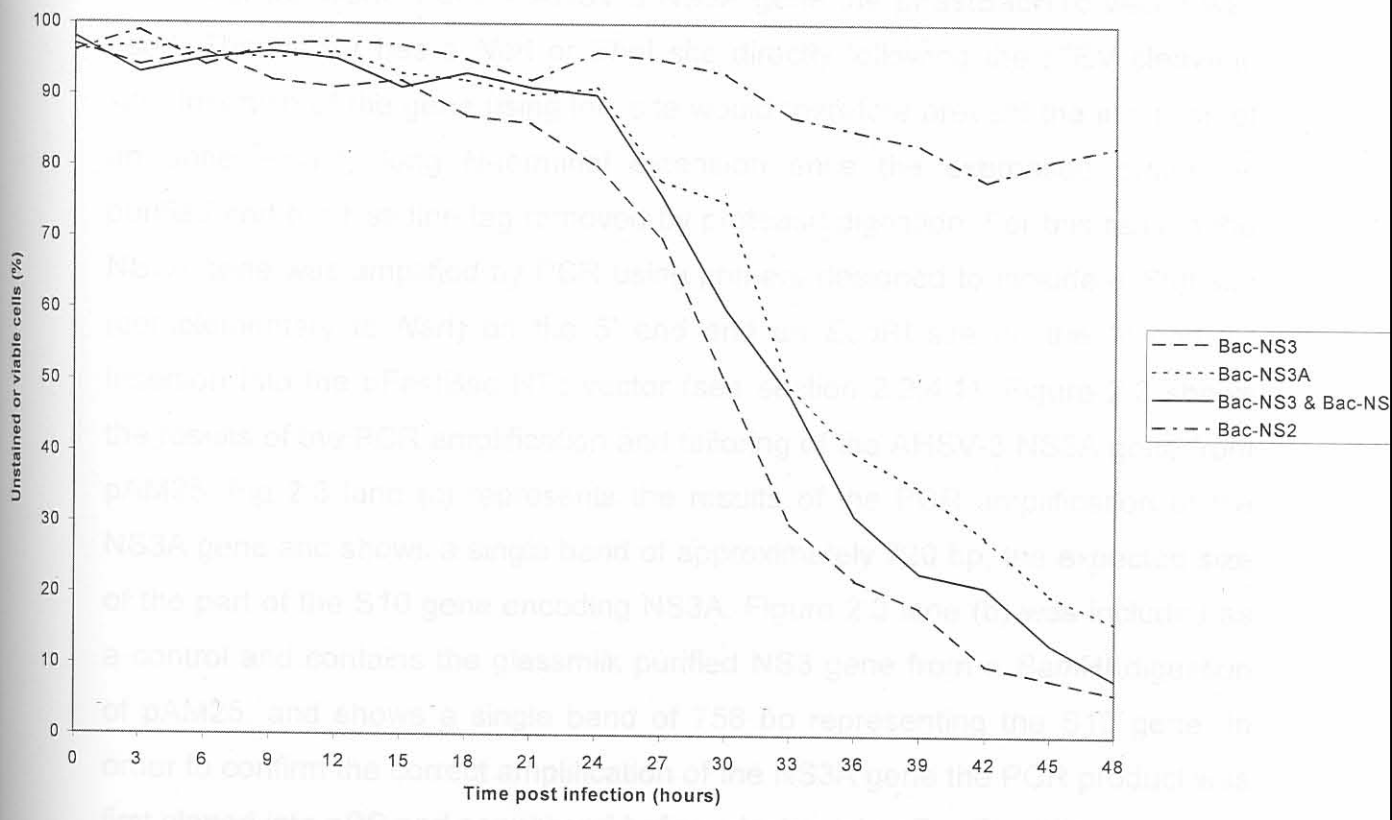


Figure 2.2 (A) Western immunoblot analysis of baculovirus expression of AHSV-3 NS3 and NS3A at 24 h.p.i. *Sf*9 cells were infected with Bac-NS2 (a), Bac-NS3 (b), Bac-NS3A or co-infected with Bac-NS3 and Bac-NS3A (d). Proteins from infected cells were separated by 15% SDS-PAGE and transferred to nitrocellulose membranes for reaction with the α-β-gal-NS3 serum. The molecular weights of the rainbow markers are indicated. **(B)** Trypan blue uptake of *Sf*9 cells infected with recombinant baculoviruses.

for Ni^{2+}-NTA (nitrilotriacetic acid) resin based on the principles of Ni^{2+}-affinity chromatography (Hochuli *et al.*, 1987). The vector also includes a rTEV protease cleavage site between the Histidine tag and the foreign protein, for the removal of the tag after purification (Parks *et al.*, 1994).

The AHSV-3 NS3 gene was modified and cloned into pBS and pFASTBAC HTc by AE Mercier, Dept Genetics UP, although the sequence of the recombinants had not been confirmed. These plasmids, pBS-NS3H and pBACHTc-NS3 were, therefore, sequenced here (results not shown) and the presence, correct reading frame for expression and integrity of the NS3 gene confirmed.

For the cloning of the AHSV-3 NS3A gene the pFastBacHTc vector was used. This vector has a *Nar*I or *Ehe*I site directly following the rTEV cleavage site. Insertion of the gene using this site would therefore prevent the inclusion of an unnecessarily long N-terminal extension once the expressed protein is purified and the histidine tag removed by protease digestion. For this reason the NS3A gene was amplified by PCR using primers designed to include a *Sfu*I site (complementary to *Nar*I) on the 5' end and an *Eco*RI site on the 3' end for insertion into the pFastBac HTc vector (see section 2.2.4.1). Figure 2.3 shows the results of the PCR amplification and tailoring of the AHSV-3 NS3A gene from pAM25. Fig 2.3 lane (c) represents the results of the PCR amplification of the NS3A gene and shows a single band of approximately 720 bp, the expected size of the part of the S10 gene encoding NS3A. Figure 2.3 lane (b) was included as a control and contains the glassmilk purified NS3 gene from a *Bam*HI digestion of pAM25, and shows a single band of 758 bp representing the S10 gene. In order to confirm the correct amplification of the NS3A gene the PCR product was first cloned into pBS and sequenced before cloning into pFastBac-HTc.

Cloning into pBS was achieved by firstly end-filling the PCR product using Klenow polymerase and then digesting the NS3A gene with *Eco*RI. The pBS vector was digested with *Sma*I, a blunt end cutter, and *Eco*RI before the vector and insert were ligated. Following ligation the possible recombinant plasmids were transformed into *E. coli* cells. Plasmid DNA was isolated from possible

recombinants and analysed by *Sfu*I and *Eco*RI digestion, which should result in the excision of the NS3A gene, if present. The results of the restriction enzyme digestions are given in Figure 2.4. Lane b shows the digestion of the pBS vector, which as expected resulted in the linearisation of the 3.2 kb plasmid. Note that incomplete digestion occurred and that the bright lower band in lane b represents super-coiled plasmid pBS DNA. Digestion of the possible recombinant in lane c (Fig. 2.4) resulted in two bands of 3.2 kb and approximately 700 bp. This is therefore a recombinant with the NS3A gene excised (700bp) and the pBS plasmid linearised (3.2 kb). Lanes (d-h) represents non-recombinant pBS plasmids.

The recombinant plasmid represented in lane c (Fig. 2.4) was digested with *Bgl*II to verify that only a single copy of the NS3A gene was inserted into the pBS vector, as this enzyme does not cleave the pBS plasmid but only the NS3A gene. The recombinant plasmid was cleaved once by this enzyme and linearised indicating the presence of one insert only (results not shown). The recombinant was termed pBS-NS3AH. The plasmid was sequenced (results not shown) and found to contain the NS3A gene and the correct restriction enzyme sites, without any modifications.

The NS3A gene was then sub-cloned into the pFastbac HTc vector. This was acheived by excision of the gene from pBS-NS3AH by *Sfu*I and *Eco*RI and digestion of the pFastbac HTc plasmid with *Nar*I and *Eco*RI, followed by ligation of vector and insert. Cleavage with the *Sfu*I and *Nar*I restriction enzymes results in complementary overhangs. Ligated plasmids were transformed into *E. coli* cells and possible recombinant plasmids purified and analysed by *Eco*RV and *Eco*RI digestion. Digestion of the pFastbac HTc plasmid with these enzymes would result in three bands of 3559 bp, 1263 bp and 33 bp. Digestion of a recombinant containing the NS3A gene would result in three bands of 3559 bp, 1263 bp and 733bp, approximately. Figure 2.5 illustrates the results obtained with 5 possible recombinant plasmids. Lane b (Fig 2.5) contains *Eco*RV and *Eco*RI digested pFASTBAC HTc DNA, only two bands (approximately 3559 and 1263 bp) are visible as the 33bp segment is too small for visualisation. Lanes c-e

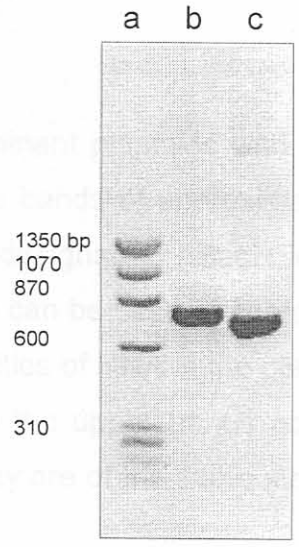


Figure 2.3 Gel electrophoretic analysis of AHSV-3 NS3A gene. The NS3A gene was PCR amplified and tailored from pAM25 (c) and compared to glassmilk purified NS3 (b) obtained from a *Bam*HI digestion of pAM25. *Hae*III-digested ΦX174 was included as molecular weight marker (a).

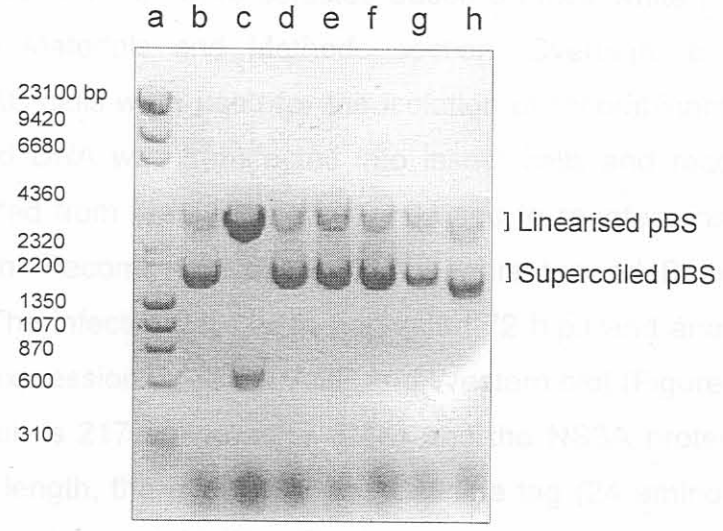


Figure 2.4 Agarose gel electrophoretic analysis of the excision of the AHSV-3 NS3A gene from possible recombinant pBS plasmids, by *Sfu*I and *Eco*RI digestion (c-h). The pBS plasmid was digested with *Sfu*I and *Eco*RI as a negative control (b). *Hin*dIII-digested λ DNA and *Hae*III-digested ΦX174 were included as molecular weight markers (a).

and g (Fig. 2.5) contain recombinant plasmids with the NS3A gene as can be seen from the presence of three bands of approximately 3559 bp, 1263 bp and 733 bp. Recombinants were designated pBacHTc-NS3A. Lane f (Fig 2.5) contained a non-recombinant as can be seen by the presence of only two bands. The presence of the large quantities of RNA in the plasmid extractions in lanes c-g (Fig 2.5) probably resulted in the upper bands occurring at a lower position than those in lane b although they are of the same size.

2.3.4 Expression of NS3 and NS3A as histidine tag fusion proteins

The pBacHTc-NS3 and pBacHTc-NS3A recombinants identified in 2.3.3 were transformed into competent DH10BAC cells for transposition into the BACMID plasmid. Recombinants were selected based on their white phenotype as described in the Materials and Methods section. Overnight cultures of recombinant DH10BAC cells were used for the isolation of recombinant Bacmid DNA. Purified Bacmid DNA was transfected into insect cells and recombinant baculoviruses harvested from the supernatant and used to re-infect insect cells for protein expression. Recombinant baculoviruses were termed BacHTc-NS3 and BacHTc-NS3A. The infected cells were harvested 72 h.p.i and analysed for recombinant protein expression by SDS-PAGE and Western blot (Figure 2.6).

The NS3 protein is 217 amino acids (24K) and the NS3A protein is 205 amino acids (23K) in length, the addition of the histidine tag (24 amino acids in total) should result in an increase in size of approximately 2K. Proteins from *Sf9* cells infected with BacHTc-NS3 have a single definite band of the expected size for HTc-NS3 (26K) (Fig 2.6, lanes d and e). Extracts from *Sf9* cells infected with BacHTc-NS3A have a single band of the expected size for HTc-NS3A (25K) (Fig 2.6, lanes f and g). The presence of the smaller bands above the NS3 and NS3A histidine tag fusion proteins could not be explained. All lanes, including mock-infected (Fig 2.6, lane b) and wild-type baculovirus infected (Fig 2.6, lane c), show a non-specific band at a position just below the 66 kDa size marker.

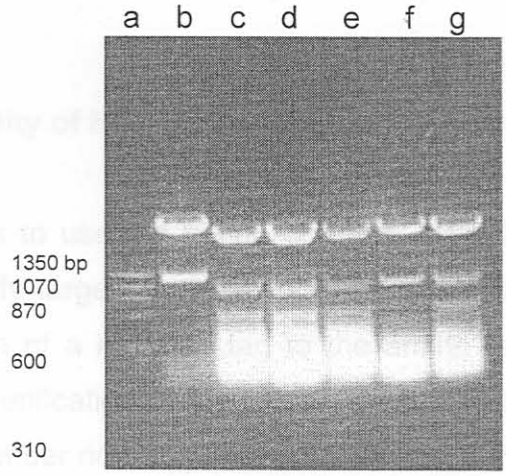


Figure 2.5 Agarose gel electrophoretic analysis of *Eco*RI and *Eco*RV digestion of possible pFastBac HTc NS3A recombinants (c-g). pFastBac HTc was digested with *Eco*RI and *Eco*RV as negative control (b). *Hae*III-digested ΦX174 included as a molecular weight marker (a).

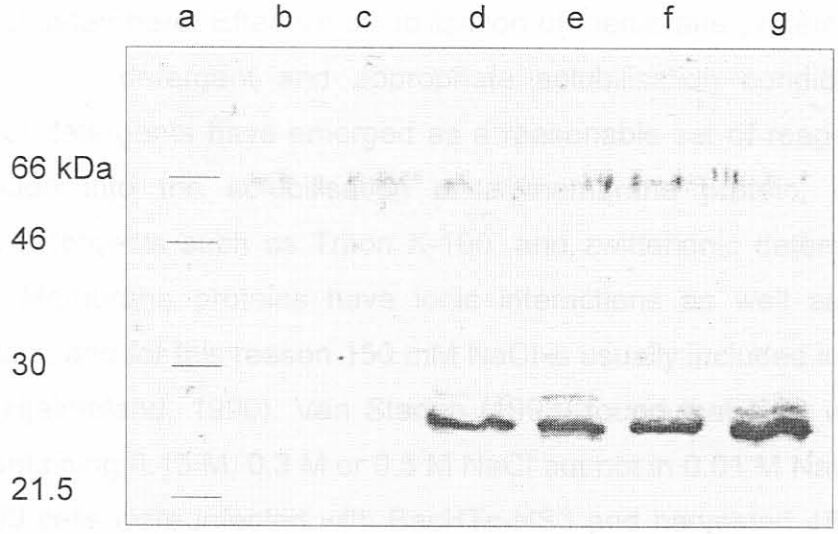


Figure 2.6 Western immunoblot of recombinant baculovirus expression of NS3 and NS3A as histidine tag fusion proteins. *Sf*9 cells were mock-infected (b) or infected with wild-type (c), BacHTc-NS3 recombinant (d,e) or BacHTc-NS3A recombinant (f,g) baculoviruses. Proteins from infected cells were separated by 12% SDS-PAGE and transferred to nitrocellulose membranes for reaction with the α-β-gal-NS3 serum. The molecular weights of the rainbow markers are indicated (a).

2.3.5 Analysis of solubility of HTc-NS3 and binding to nickel resin

The aim here was to use the fusion proteins, HTc-NS3 and HTc-NS3A, produced above, to purify large quantities of NS3 and NS3A in a biologically active form. The addition of a histidine tag to the amino terminus of a protein allows for the one-step purification of the protein on Ni-nitrilo-tri-acetic acid resin columns (Ni-NTA-resin) under non-denaturing conditions, if the protein is soluble. Highly insoluble proteins can only be purified following treatment with denaturing agents such as SDS, which lead to the disruption of the structure or native conformation of the protein with the result that the protein is no longer in a biologically active form. For this reason the solubility of the HTc-NS3 protein was first investigated here. Effective solubilisation of membrane proteins requires the selection of a detergent and appropriate solubilisation conditions. A small number of detergents have emerged as a reasonable set of reagents for a first investigation into the solubilisation of a membrane protein, these include nonionic detergents such as Triton X-100, and zwitterionic detergents such as CHAPS. Membrane proteins have ionic interactions as well as hydrophobic interactions, and for this reason 150 mM NaCl is usually included in solubilisation buffers (Hjelmeland, 1990). Van Staden (1993) found that NS3 was soluble in buffer containing 0.15 M, 0.3 M or 0.5 M NaCl but not in 0.01 M NaCl.

Sf9 cells were infected with BacHTc-NS3 and harvested 48-72 h.p.i., the cells were then disrupted in lysis buffer A (2.2.6.1) adjusted to contain 0.1, 0.25, 0.5, 1.0 or 2.0% Nonidet P-40, a nonionic detergent. Following lysis the cell extracts were separated into soluble (S) and insoluble or pellet (P) fractions by centrifugation. Proteins in each fraction were analysed by SDS-PAGE and Western immunoblot with α-β-gal-NS3 serum, the results are shown in Figure 2.7. As can be seen from Fig 2.7 the most effective Nonidet P-40 concentration was 1.0%, with approximately 50% of the protein solubilised. At 0.1%, 0.25% and 0.5% Nonidet P-40 less than 50% of the HTc-NS3 protein was solubilised, while the protein was completely insoluble in buffer containing 2.0% Nonidet P-40. A non-specific protein band, migrating just below the 66 kDa size marker,

was observed in each lane, this band is commonly seen throughout and represents a non-specific protein recognised by the α-β-gal-NS3 serum.

In order to improve these results another nonionic detergent was investigated, Triton X-100. The solubilisation assay was performed as before with lysis buffer A adjusted to contain 0.1, 0.25, 0.5, 1.0 or 2.0% Triton X-100. Figure 2.8 shows the results obtained with 0.5, 1.0 and 2.0% Triton X-100. As a control the pellet and soluble fractions after treatment with buffer A with 1.0% Nonidet P-40 were included. Treatment with 1.0% Triton X-100 appeared to improve the solubility of the histidine tagged protein, with approximately 60%, repeatably occuring in the soluble fraction. The HTc-NS3 protein was insoluble at 2.0% Triton X-100 and only partly soluble in 0.5% Triton X-100.

Next the optimal salt concentration for solubilisation was determined. BacHTc-NS3 infected *Sf9* cells were lysed as before in lysis buffer A with 1.0% Triton X-100 and 0.15 M, 0.3 M or 0.5 M NaCl. Soluble and pellet fractions after centrifugation were analysed as before (Figure 2.9). The HTc-NS3 protein was 60-80% solubilised in the presence of 0.3 M NaCl and 1.0% Triton X-100. At 0.15 M and 0.5 M NaCl the solubility of HTc-NS3 was significantly lower.

The ability of the solubilised HTc-NS3 to bind to the Ni-NTA-resin was then investigated. Binding of the histidine tag to the Nickel resin may be influenced by the concentration of the imidazole in the wash buffers (GIBCO BRL BAC-to-BAC^{TM} Baculovirus expression manual). The recommended concentration is 20 mM, but decreasing the concentration to between 0-15 mM may significantly improve binding. Two imidazole concentrations in the wash buffer were investigated here, namely 10 mM and 20 mM. The results are shown in Fig 2.10. Briefly, solubilised HTc-NS3 (Fig 2.10, S) was mixed with the charged nickel resin and binding allowed to occur for 30 min. The resin and bound proteins were collected by centrifugation and unbound proteins (Fig 2.10, c,g) retained. The resin was then washed in wash buffer (containing 10 or 20mM imidazole) and collected, while proteins in the supernatant were retained (Fig 2.10, d,h). Bound proteins were then eluted in the presence of elution buffer (containing a high concentration of imidazole)(Figure 2.10, lanes a,e), this step

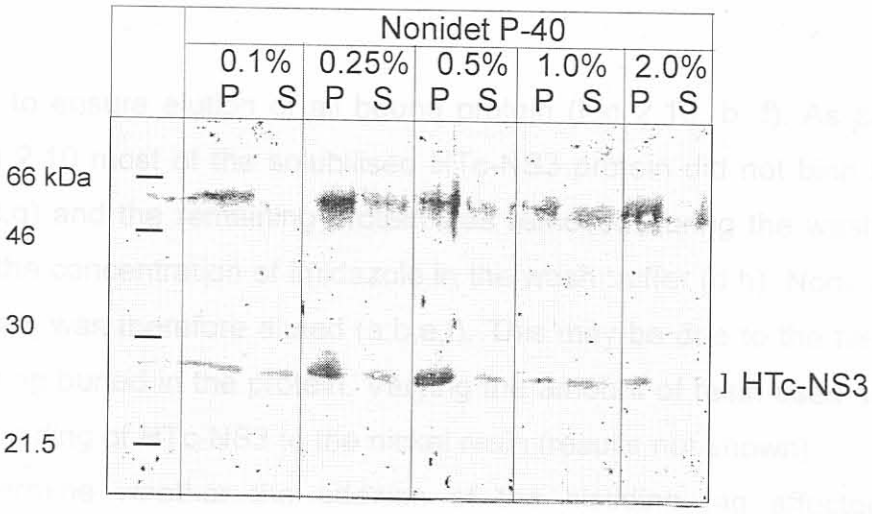


Figure 2.7 Western immunoblot analysis of solubility of HTc-NS3 in lysis buffer A adjusted to contain the indicated concentrations of the nonionic detergent Nonidet P-40. P – pellet fraction containing insoluble proteins, S – supernatant fraction containing solubilised proteins. The molecular weights of the Rainbow markers are indicated

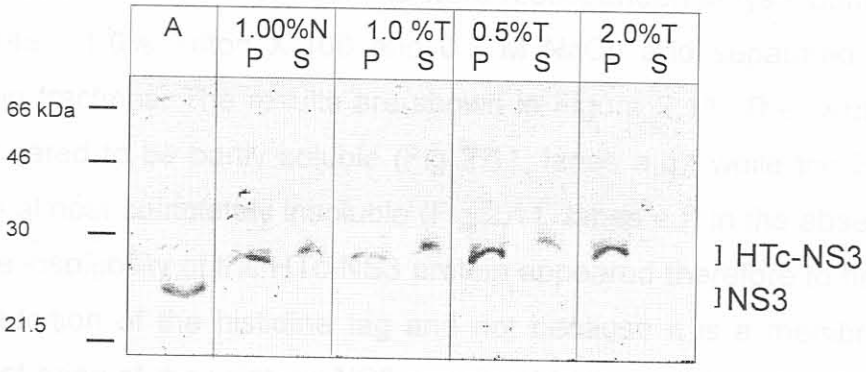


Figure 2.8 Western immunoblot analysis of solubility of HTc-NS3 in lysis buffer A adjusted to contain the indicated concentrations of the detergent Triton X-100. The molecular weight of the rainbow markers are indicated. Proteins from *Sf*9 cells infected with Bac-NS3 were included as a control (A). N-Nonidet P-40, T-Triton X-100.

was repeated to ensure elution of all bound protein (Fig 2.10, b, f). As can be seen from Fig 2.10 most of the solubilised HTc-NS3 protein did not bind to the nickel resin (c,g) and the remaining protein was removed during the wash step regardless of the concentration of imidazole in the wash buffer (d,h). None of the HTc-NS3 protein was therefore eluted (a,b,e,f). This may be due to the histidine residue tag being buried in the protein. Varying the amount of resin used did not influence the binding of HTc-NS3 to the nickel resin (results not shown).

To determine whether the addition of the histidine tag affected the solubility of NS3, the solubility of the HTc-NS3 fusion protein was compared to that of wildtype NS3. *Sf9* cells were infected with Bac-NS3 or BacHTc-NS3 and harvested 48 h.p.i. The cells were then resuspended and lysed in lysis buffer A (without detergent) and separated into soluble and insoluble fractions by centrifugation. Proteins in the pellet fractions were resuspended in lysis buffer A (adjusted to contain 1.0% Triton X-100 and 0.3 M NaCl) and separated into pellet and soluble fractions. The results are shown in Figure 2.11. The wildtype NS3 protein appeared to be partly soluble (Fig 2.11, lanes a,b), while the HTc-NS3 protein was almost completely insoluble (Fig 2.11, lanes e,f) in the absence of detergent. The insolubility of the HTc-NS3 protein appeared therefore to be as a result of the addition of the histidine tag and not because it is a membrane protein. As almost none of the wildtype NS3 remained in the pellet no bands are visible in lanes c and d (Fig 2.11). The pellet fraction in lane e (Fig 2.11), i.e insoluble HTc-NS3, was resuspended in lysis buffer with detergent and, as was found previously, approximately 60-80% of the protein was now solubilised. The insolubility of the protein may therefore be more a function of incorrect folding due to the presence of the histidine tag. It was therefore decided that the HTc-NS3 protein would not be suitable for large scale purification of NS3.

2.3.6 Sequence analysis of the N-terminal region of NS3

As yet no functional differences between AHSV NS3 and NS3A have been reported. The presence of a functional domain in the N-terminal region of NS3, that is absent in NS3A, could potentially result in a functional difference

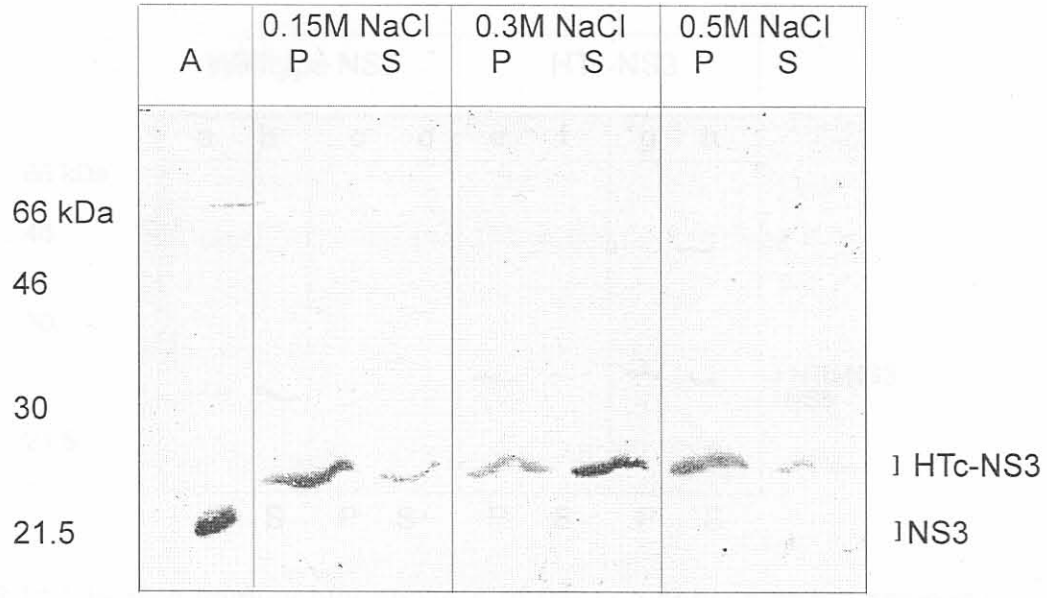


Figure 2.9 Western immnuoblot analysis of solubility of HTc-NS3 in lysis buffer A adjusted to contain 1.0% Triton X-100 and the indicated concentrations of NaCl. Proteins from *Sf*9 cells infected with Bac-NS3 were included as a control (A). The molecular weights of the rainbow size markers are indicated.

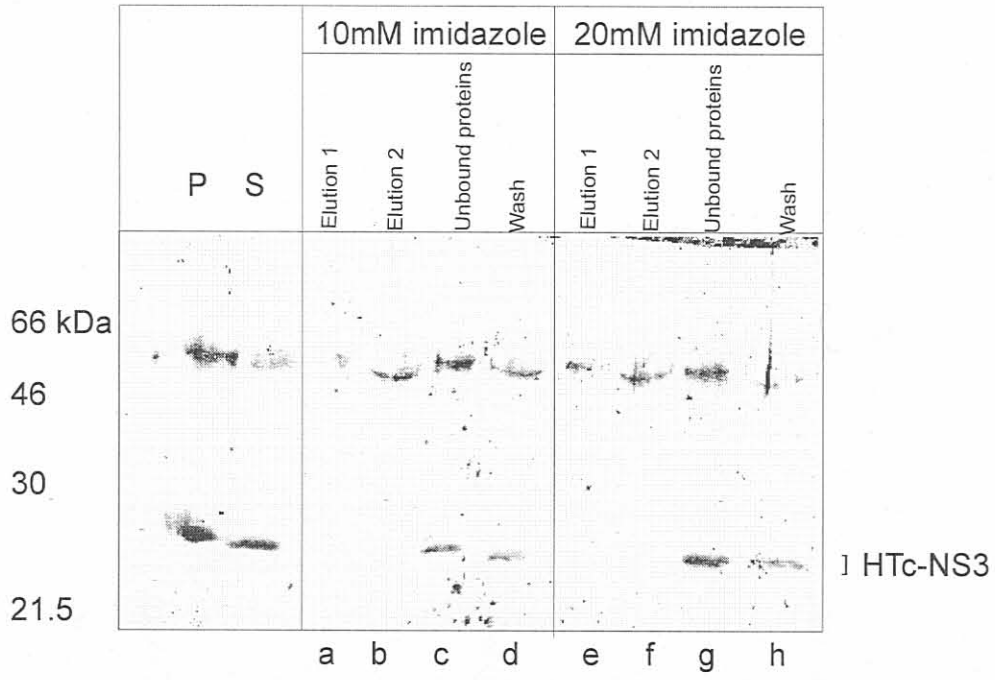


Figure 2.10 Western immunoblot analysis of the binding of the HTc-NS3 protein to Ni-NTA resin. The molecular weights of the size markers are indicated.

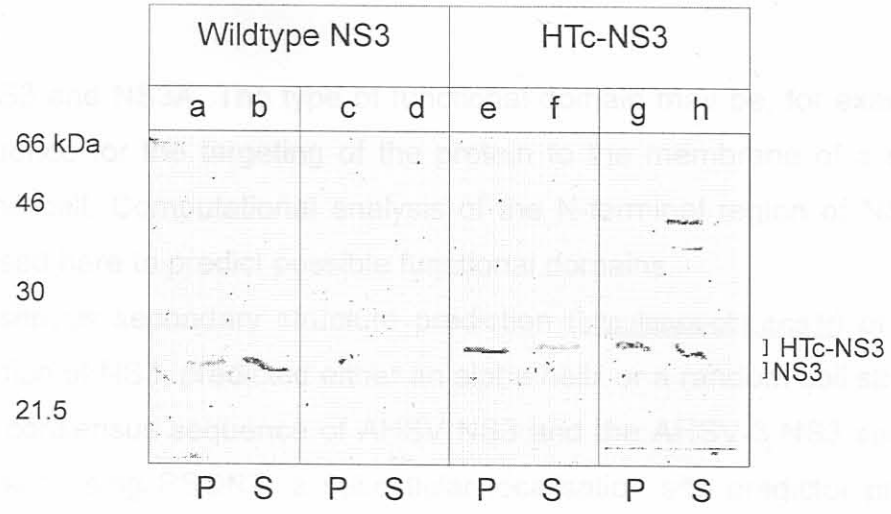


Figure 2.11 Western immunoblot analysis of the comparison of the solubility of HTc-NS3 and wildtype NS3. *Sf*9 cells infected with Bac-NS3 or BacHTc-NS3 were lysed in lysis buffer A and separated into pellet (P)(a and e) and supernatant fractions (S)(b and f). Proteins in pellet fractions were then collected and resuspended in lysis buffer A (adjusted to contain 1.0% Triton X-100 and 0.3M NaCl) and separated into pellet (c and g) and supernatant fractions (d and h). Size markers are indicated.

between NS3 and NS3A. The type of functional domain may be, for example, a signal sequence for the targeting of the protein to the membrane of a specific region in the cell. Computational analysis of the N-terminal region of NS3 was therefore used here to predict possible functional domains.

Consensus secondary structure prediction (http://npsa-pbil.ibcp.fr) of the N-terminal region of NS3, predicted either an alpha helix or a random coil structure.

The consensus sequence of AHSV NS3 and the AHSV-3 NS3 sequence were analysed using PSORT, a subcellular localisation site predictor program. The AHSV-3 NS3 PSG score was –1.74 and the GvH score –5.11 and the protein predicted as not having a signal peptide. iPSORT analysis of the first 10amino acids of NS3 and the first 30 amino acids of NS3A predicted the absence of a signal targeting peptide. The NS3 proteins of some of the Orbiviruses (Table2.1, discussed later), furthermore, contain one or more acidic amino acids, a common feature of signal sequences being the absence of acidic amino acids.

As the N-terminal region may contain a functional domain for another purpose than a signal peptide, the NS3 N-terminal residues were also scanned against protein profile databases (including Prosite) (http://isrec.isb-sib.ch/). The sequence was also analysed using PattinProt (http://npsa-pbil.ibcp.fr) which scans a protein sequence or a protein database for one or several patterns. Neither pattern nor sequence searches revealed significant patterns or profiles (results not shown).

If a functional domain were present in this region the domain would be expected to be relatively conserved. The amino acid sequence of the N-terminal region of NS3 of various orbiviruses and the different AHSV serotypes were compared (Table 2.1). The amino acid sequence of this region in the different serotypes of AHSV NS3 were found not to be highly conserved either in amino acid sequence or in length. The two in-phase methionines were separated by either 9 or 10 amino acids, and only four of these were conserved in all serotypes. When compared to the NS3 proteins of other orbiviruses, such as BTV, EHDV, Broadhaven (BRD) and Palyam (Table 2.1) it can be seen that this

Table 2.1 Amino acid sequences of the N-terminal regions of the NS3 proteins of AHSV, Palyam virus, Broadhaven (BRD) virus, BTV and Epizootic haemorrhagic disease virus (EHDV)

Virus	Sequence
AHSV-1[1], AHSV-2[2],AHSV-8[2]	*MNLASISQSYMS
AHSV-3[3], AHSV-7[2]	MSLATIAENYMM
AHSV-4[2], AHSV-5[2], AHSV-6[4], AHSV-9[3]	MNLAAIAKNYSM
AHSV consensus	M‡ZLA†XIXXZY(X)M
Palyam virus[5]	MLARSLNEYKAM
BRD[6]	MLAALEM
BTV-1,2,10,11,13,17[7]	MLSGLIQRFEEEKM
EHDV[8]	MLSRLVPGVETRIEM

[1] De Sa *et al.*, 1994, [2] Martin *et al.*, 1998, [3] Van Staden & Huismans 1991, [4] Van Niekerk *et al.*, 2001b, [5] De Sa *et al.*, 1994, [6] Moss *et al.*, 1992, [7] Hwang *et al.*, 1992

[8] Jensen *et al.*, 994

*Deduced translation initiation sites for NS3 and NS3A are underlined.

†X indicates any amino acid, ‡Z indicates similar amino acids in the AHSV consensus sequence.

region varies greatly in length and sequence between the different viruses. The number of amino acids between the two methionines ranged from 5 to 13. It should be noted however that the presence of the second methionine or initiation site is conserved between these orbiviruses.

2.4 DISCUSSION

The central aims of this part of the study were to compare the NS3 and NS3A proteins of AHSV and to further characterise the cytotoxic properties of these proteins.

AHSV NS3 is postulated to be related to a family of viral proteins, viroporins, due to the presence of characteristic structural features within the protein and its cytotoxic effect on insect cells when expressed endogenously (Van Staden *et al.*, 1995, Van Staden *et al.*, 1998, Van Niekerk *et al.*, 2001a). The cytotoxic effect of the protein to mammalian cells has not yet been investigated and the protein has not been expressed as a recombinant in mammalian cells.

An alternative to the endogenous expression of the protein in mammalian cells would be the exogenous addition of the purified protein to cultured cells and analysis of the cytotoxic effect. A large number of viroporins have been shown to cause cell membrane permeabilisation when added extracellularly to cell cultures. This toxic effect may occur in much the same manner as natural cytolytic proteins or peptides, such as magainins, cecroporins and melittin, by forming hydrophilic pores through cellular membranes, leading to osmotic disintegrity (Miller *et al.*, 1991, Miller & Montelaro 1992) or via a receptor-mediated mechanism. A number of examples of proteins which display this characteristic include rotavirus NSP4 (Tian *et al.*, 1996b), HIV-1 Vpr (Macreadie *et al.*, 1996; Piller *et al.*, 1998), HIV-1 transmembrane protein (Miller *et al.*, 1993), HIV-1 gp120 (Annunziata *et al.*, 1998), SIV SU envelope glycoprotein (Swaggerty *et al.*, 2000) and vesicular stomatitus (VSV) glycoprotein (Schlegel & Wade 1985). The effect of AHSV NS3 on Vero cell membranes following extracellular addition was, therefore, investigated here.

Sf9 cell extracts from Bac-NS3 and wild-type baculovirus infected cells were prepared as outlined in section 2.3.1. These NS3-containing and wild-type baculovirus extracts were added to Vero cells and the effect on the membrane monitored via the uptake of the membrane impermeable translation inhibitor,

Hyg B. The NS3 protein was found to cause an increase in the uptake of Hyg B from the environment, indicative of an increase in the permeability of the plasma membranes of the Vero cells. The AHSV NS3 protein, therefore, appears to not only be cytotoxic to insect cells, but also causes membrane permeabilisation of Vero (mammalian) cells. This extends the idea that NS3 is a viroporin and can form the basis of future experiments as will be detailed in chapter 4.

The mechanism of NS3 membrane permeabilisation needs to be investigated further. To facilitate this the purification of biologically active NS3 and NS3A was attempted, as will be described later.

AHSV-3 infection of Vero cells results in the expression of both NS3 and NS3A, 13 to 24 hpi, in approximately equimolar amounts (Van Staden *et al.,* 1995). When the full length AHSV-3 segment 10 gene was, however, expressed in the baculovirus system in insect cells, only the NS3 protein (24K) was detected, while the NS3A (23K) protein appeared to be absent (Van Staden *et al.,* 1995). The AHSV S10 gene, therefore, displays different expression patterns in the different cell types. Recombinant baculovirus expression of segment 10 of BTV, in contrast, results in the synthesis of both NS3 and NS3A (French *et al.,* 1989). These results may be explained in the light of recent experimental findings of Chang *et al.,* (1999) who examined the roles of nucleotides flanking a baculovirus AUG initiator codon in modulating translation initiation in lepidopteran insect cells. Based on their findings, substitutions at position –3 (relative to the A of the initiator AUG codon, which was designated +1) did not appear to affect translational initiation efficiency, even though this is a highly conserved position in eukaryotic systems. The +4 and +5 positions were identified as most critical in modulating translation initiation efficiency, with the optimal initiator context being, 5'-NNNAUG**A(A/C/G)**N-3'.

Translation initiation of two proteins from the same mRNA occurs via a context-dependant leaky scanning mechanism (Kozak 1991). When the first AUG is in a sub-optimal context, some ribosomes are allowed to continue scanning along the mRNA and initiate translation at the following AUG, which is in a more favourable context.

The initiation contexts of the NS3 and NS3A genes of AHSV and BTV are given in Table 2.2. In the AHSV-3 S10 gene the first AUG is in a more favourable context for expression in insect cells, according to Chang *et al.,* (1999), than that for the initiation of NS3A. In the BTV S10 gene, however, the NS3A initiation codon is in an optimal context, while that for the NS3 is weaker. This may explain why both proteins are synthesised in insect cells when the S10 gene of BTV is expressed as a baculovirus recombinant, but only NS3 of AHSV-3 is expressed when the same strategy is followed. Expression of AHSV-2 S10 in insect cells, furthermore, also results in the synthesis of NS3 alone (M van Niekerk personal communication) this can also be explained in terms of the initiation context of the two translation start codons.

Both NS3 and NS3A are cytotoxic to insect cells when expressed individually (Van Staden *et al.,* 1995; Van Niekerk *et al.,* 2001a). Expression of the two proteins together in insect cells, as observed in AHSV-infected cells, may potentially lead to interactions between the proteins that affect their cytotoxicity. This has not previously been studied and was, therefore, investigated here.

In this study the co-expression of AHSV-3 NS3 and NS3A was achieved in insect cells by co-infection of cells with recombinant baculoviruses expressing NS3 and NS3A. Another approach for the co-expression of two proteins would be the use of a vector (such as pFastBac Dual) that allows for the cloning and expression of two genes. The baculovirus system depends on site-specific transposition of foreign genes in transfer vectors to the Bacmid DNA. The presence of two almost identical genes, such as NS3 and NS3A, may however interfere with this transposition process. This was therefore not investigated here. Alternatively the initiation codon context surrounding the NS3 and NS3A start codons could be modified in the full length S10 gene.

The cytotoxic effect on insect cells of endogenous expression of both NS3 and NS3A, together, was then investigated. The viability of *Sf9* cells was monitored via the uptake of the vital exclusion dye, Trypan blue. Whether expressed together or individually, the NS3 and NS3A proteins cause a rapid increase in cell death from 24 to 48 h.p.i., at which time only 6-20% of the cell

Table 2.2 Translation initiation codon context for the expression of NS3 and NS3A from S10 genes of various AHSV serotypes and BTV.

Virus	Initiation codon context	
	NS3	**NS3A**
AHSV-3[1]	5'-AUG**AG**U.....	.....AUG**AU**G-3'
AHSV-2[2]	5'-AUG**AA**U.....	.....AUG**UC**A-3'
AHSV-9[1]	5'-AUG**AA**U.....	.....AUG**CA**U-3'
BTV[3]	5'-AUG**CU**A.....	.....AUG**AA**A-3'

[1]Van Staden & Huismans 1991
[2]Van Niekerk *et al.*, 2001b
[3]Hwang *et al.*, 1992

Initiation codons are underlined
+4 and +5 positions are in bold

population remain viable. The cytotoxic effect of either protein individually was therefore similar to the effect when expressed together. There appears to be no interaction between the two proteins that influences this aspect of their functioning. The NS3 protein is therefore representative of NS3 and NS3A in terms of its cytotoxic effect on cells.

The availability of large amounts of purified NS3 and NS3A would facilitate further investigations into the function and role of these proteins in AHSV infection. This part of the study dealt with the expression of the segment 10 genes as histidine tag fusion products in the baculovirus system.

The baculovirus expression system allows for the production of high levels of a foreign protein, in a native form in an eukaryotic system. Expression of the foreign proteins as histidine tag fusion proteins allows for their purification through the strong affinity of the histidine tag for charged Nickel resin (Hochuli *et al.,* 1987; Crowe *et al.,* 1995). Following purification the histidine residues can be removed by rTEV protease cleavage (Parks *et al.,* 1994) with the release of the protein in a biologically active form. The PsaD (peripheral stromal-facing subunit of photosystem I) protein (Minai *et al.,* 2001), the chloroplast triose phosphate-phosphotase translocator protein (Loddenkotter *et al.,* 1993), the Hepatitis B virus preS domains (Nunez *et al.,* 2001), the Rubella virus capsid protein (Schmidt *et al.,* 1998) and the Japanese encephalitis virus NS3 protein (Kuo *et al.,* 1996) were all successfully purifed using this single-step affinity chromatography method. Westra and co-workers (2001) furthermore report the successful purification of four different histidine-tagged recombinant proteins expressed in insect cells.

For the cloning of the AHSV-3 NS3A gene into the pFastBac HT vector, the gene was PCR tailored from a cloned copy of the S10 gene. Primers were designed to amplify only the NS3A gene (i.e. from the second start codon) and to install a *Sful* site at the 5' and an *Eco*RI site at the 3' ends of the gene. The tailored NS3A gene was cloned into pBS and the presence and sequence of the gene confirmed. Next, the NS3A gene was subcloned into pFastBacHTc and the insertion verified by restriction enzyme digestion. The AHSV-3 NS3 gene was

cloned, using a similar strategy, into pBS and pFastBacHTc by AE Mercier. The sequence of the PCR amplified NS3 gene was confirmed here by automated sequencing analysis.

The pBacHTc-NS3 and pBacHTc-NS3A plasmids were used to produce recombinant baculoviruses that express NS3 and NS3A, respectively, as histidine fusion proteins. Western blot analysis of proteins from insect cells infected with these baculoviruses revealed the presence of the fusion proteins (26 kDa HTc-NS3 and 25 kDa HTc-NS3A). Purification of the recombinant fusion proteins, in a nondenatured form, on Ni-NTA resin columns necessitates their solubilisation. The solubility of the HTc-NS3 protein was therefore investigated and optimised here. Maximal solubilisation (60 - 80%) was achieved under conditions of 1.0% Triton X-100, a nonionic detergent, and 0.3 M NaCl. The soluble protein was shown, however, not to bind to the charged nickel resin. Variation of the conditions under which binding occurred did not appear to improve or effect binding, so it was postulated that the histidine tag may be buried within the protein. The wildtype NS3 and HTc-NS3 proteins were compared in terms of their solubility. The addition of the histidine tag to the protein was found to significantly decrease the solubility of NS3. The insolubility of the protein was not due to the membrane associating properties of NS3 as was initially thought. It was decided that the HTc-NS3 protein would not allow for the purification of NS3 in a native form.

Similar findings are reported by Rumlova *et al.,* (2001), who compared classical and histidine-tagged affinity purification techniques, in terms of their applicability in the purification of the Mason-Pfizer monkey virus capsid protein. They showed that the widely used histidine anchor may significantly alter the properties of the protein of interest, in particular the structure or morphology of the protein was dramatically influenced. Lasnik and co-workers (2001), furthermore report that the only efficient way to obtain biologically active protein (human granulocyte colony stimulating factor) was through complete denaturation with guanidine-HCl or urea and subsequent renaturation.

No functional difference between AHSV NS3 and NS3A has been described. As the presence of a functional domain in the N-terminal region of NS3, that is absent in NS3A, may result in a functional difference between these proteins, the amino acid sequence of this region was analysed. Computer predictions and searches where performed to identify any putative functional domains, such as signal sequences, or patterns and motifs.

The N-terminal region of NS3 was first computationally analysed for the presence of a signal sequence that may lead to the differential localisation of the NS3 and NS3A proteins. Signal or leader sequences are usually found at the N-terminus of a protein, and are generally 15-30 amino acids in length. At or close to the N-terminus of signal sequences there are 2-3 polar residues, and within the leader is a hydrophobic core consisting exclusively or very largely of hydrophobic amino acids. There is no other conservation of sequence. There are no acidic groups in the leader, and usually its net charge is approximately +1.7. The leader sequence of proteins targeted to organelles such as the mitochondrion also have little homology. The leaders are usually between 12 and 30 amino acids in length, hydrophilic, consisting of stretches of uncharged amino acids interrupted by basic amino acids and they lack acidic amino acids (Lewin 1994). Membrane proteins may or may not contain a leader sequence and are retained in the membrane by transmembrane regions within the protein.

Sequence analysis of the N-terminal region of NS3 revealed the absence of potential signal sequences. Scanning of this region against protein databases and profiles did not identify any significant patterns or motifs. The N-terminal region of the NS3 protein was furthermore found not to be highly conserved between the different serotypes of AHSV (see Table 2.1). The N-terminal region of the NS3 proteins of related orbiviruses were also found to differ significantly in length and sequence (Table 2.1). The presence of the second methionine (or start codon) was however conserved in all the orbiviruses analysed.

The availability of antibodies directed against the N-terminal residues of NS3 would allow for the specific detection of NS3 and facilitate experimental

analyses of possible functional and localisation differences between NS3 and NS3A. This was investigated and will be discussed in the following chapter.