CHAPTER 3

EXPRESSION OF THE GENE ENCODING NON-STRUCTURAL PROTEIN NS3 OF EEV BRYANSTON (EEV-1)

3.1. INTRODUCTION

A number of conserved features or characteristics have been identified in the EEV NS3 protein in the preceding chapter, and similar features have previously been identified in other orbivirus NS3 proteins. In order to further study and characterize EEV NS3, specifically regarding the relationship between structure and function, it is therefore necessary to express the protein and to study its expression.

The NS3 genes of BTV, AHSV and EHDV have been demonstrated *in vitro* to code for two gene products, NS3 and NS3A (Van Dijk and Huismans, 1988; Van Staden and Huismans, 1991; Jensen *et al.*, 1994). Cloning and sequencing of these genes revealed the presence of two in-phase initiation codons, which could be responsible for the independent translation initiation of these two proteins. In the case of BTV and AHSV the NS3 and NS3A proteins are synthesized late in the infection cycle and are then only present at low concentrations *in vivo*, making it time-consuming or impossible to purify large amounts of protein via this route. More detailed information on the structure and function of NS3 and NS3A could only be obtained after expression of the proteins in yeast, insect and mammalian cells (e.g. French *et al.*, 1989; Hwang *et al.*, 1992; Hyatt *et al.*, 1993; Van Staden *et al.*, 1995; Stoltz *et al.*, 1996).

At present no data is available regarding the expression of EEV NS3 using *in vitro* or *in vivo* methods of expression. The first part of this chapter is therefore dedicated to the *in vitro* translation of the dsRNA of EEV Bryanston (EEV-1) alone and in comparison with AHSV, BTV and EHDV, while the second part of the chapter focuses on the expression of the EEV Bryanston (EEV-1) NS3 gene in an eukaryotic expression system. This should allow us to determine the nature of the segment 10 translation products and specifically whether the ability of BTV and AHSV segment 10 to express two proteins are conserved in EEV segment 10. The expression of the EEV Bryanston (EEV-1) NS3 gene product in an expression system will further allow the comparison of the mode of expression and expression levels to what has previously been observed for AHSV and BTV, and will establish a system to express and possibly purify NS3 of EEV in sufficient quantities to enable future functional studies (e.g. the study of post-translational modifications, and intracellular transport of proteins, and analysis of the properties of normal versus mutant proteins).

Various expression systems, prokaryotic and eukaryotic, are available for this purpose. Prokaryotic expression systems have the advantage that production can easily be scaled up to large volumes and

their host cells can be cultured continuously in relatively cheap medium, but in general such systems do not perform co-translational and post-translational modifications which are essential when immunogenic material suitable for production of antibodies is required, or if structure-function relationships are to be studied (King and Possee, 1992). Eukaryotic expression systems are capable of performing a number of post-translational modifications (e.g. glycosylation; fatty acid acylation (palmitylation and myristylation); nuclear transport; phosphorylation; C-terminus amidation; disulphide bond formation; proteolytic processing (including signal peptide cleavage, cellular targeting and secretion) and the formation of tertiary and quaternary structures), which would be found if the proteins were synthesized in the normal eukaryotic cellular environment (King and Possee, 1992).

The expression system of choice was the baculovirus expression system, an eukaryotic system, for which the techniques for cloning and expressing genes is well established in our laboratory. This expression system utilizes the baculovirus *Autographa californica* nuclear polyhedrosis virus (AcMNPV) and insect cells. It has been used to express a wide range of proteins from many sources, including virus structural and non-structural proteins. The proteins are expressed to high levels and are accurately processed and biologically active, and in most cases the proteins have also proved to be antigenic (King and Possee, 1992).

Other advantages of the baculovirus expression system also include the fact that the foreign genes are under control of a strong late promoter, and are expressed after maturation of infectious virus particles (King and Possee, 1992). Consequently, a cytotoxic protein will not adversely affect virus replication. Baculovirus genomes can also accommodate large amounts of foreign DNA without affecting normal replication and DNA packaging, and can be propagated in a variety of insect cell lines (King and Possee, 1992). Baculoviruses are safe to work with as they have a restricted host range, and insect cells are amenable to large scale volume production in fermenter systems which allows the scale up off protein production (King and Possee, 1992).

3.2. MATERIALS AND METHODS

3.2.1. Cells and viruses

The origin of the EEV Bryanston serotype (EEV-1) (a laboratory reference strain) (S1REF*), the EEV Bryanston (EEV-1) (S1REF) reference strain and the EEV Bryanston (EEV-1) (S1FLD3) field isolate was described in section 2.2.1. All the available information for the laboratory strain, reference strain and field isolate was shown in Tables 2.1., 2.2. and 2.3.

Double-stranded RNA isolated from EEV Bryanston (EEV-1) (S1REF*) was used to synthesize a cDNA copy of genome segment 10 used for cloning into pFASTBAC1 (pFB.EEVB.S10).

Other viruses used in experimental procedures include AHSV M322/97 (AHSV-3), EHDV-1, and BTV-10. In the case of AHSV M322/97, EHDV-1 and BTV-10, purified dsRNA for each was obtained from Mr. M. K. Lombardi (Department of Genetics, University of Pretoria, South Africa). The origin and history of the AHSV M322/97 isolate has been described in Van Niekerk *et al.* (2001b). AHSV M322/97 was a serotype 3 field isolate, originally obtained from the OIE Reference laboratory at the Onderstepoort Veterinary Institute (OVI), South Africa and was isolated from the spleen of dead dog in Gauteng, South Africa in 1997.

3.2.2. *In vitro* translation

In vitro translations were performed using the rabbit reticulocyte lysate system (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Generally single-stranded RNA (mRNA) is used for *in vitro* translation, but double-stranded RNA can also be used as a template (McCrae and Joklik, 1978).

Samples containing 0.5 - 2 µg of dsRNA were denatured by adding an equal volume of 10 mM MMOH and incubating at room temperature for 15 min (Mertens *et al.*, 1984). The *in vitro* translation reaction mixtures contained 0.5 - 2.0 µg denatured RNA, 8% (v/v) 12.5 x translation mix (25 mM DTT, 250 mM HEPES, pH 7.6, 100 mM creatine phosphate, 19 amino acids (312.5 µM each) minus the appropriate amino acid) (4µl of translation mix minus methionine), 100 mM KAc (2µl of 2.5M KAc solution), 0.5 mM MgAc (1 µl of 25mM MgAc solution), 40% (v/v) rabbit reticulocyte lysate (supplemented by calf liver tRNA, EGTA, creatine phosphokinase and hemin) (20 µl), RNase free water to the desired final volume of 25 or 50 µl and 10 - 15 mCi/ml (4 µl) [^{35}S]-methionine (EASYTAG™ METHIONINE L – [^{35}S]) (NEW™ Boston, MA, USA). The reactions were incubated at 30°C for 75 min and samples were stored at -20°C until use. Protein expression was analysed on 12% or 15% denaturing polyacrylamide gels (section 3.2.3.).

3.2.3. Polyacrylamide gel electrophoresis

In vitro translation protein samples were prepared for SDS-PAGE by removing 5 µl of the translation reaction and diluting with 50 µl 2 x PSB (0.125 M Tris, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.002% bromophenol blue), while protein samples from baculovirus infected *Sf*9 cells were treated with an equal volume of 2 x PSB. All protein samples were denatured by boiling at 95°C for 3 - 4 min before use.

SDS-PAGE was carried out as described by Laemmli (1970). The stacking gels contained 5% acrylamide, 0.125 M Tris, pH 6.8 and 0.1% SDS, and the separating gels 12% or 15% acrylamide, 0.375 M Tris, pH 8.8 and 0.1% SDS. The gels were prepared from a 30% acrylamide/0.8% bisacrylamide stock solution and polymerized by addition of TEMED and 10% ammonium persulphate. Electrophoresis was performed in TGS electrophoresis buffer (0.025 M Tris, pH 8.3, 0.192 M glycine, 0.1% SDS) using either the Mighty Small™ II SE 250 system (Hoefer Scientific Instruments) for 3.5 h at 120V, or the Sturdier SE 400 vertical slab gel units (Hoefer Scientific Instruments) for 16 h at 80V. Gels were either stained in 0.125% Coomassie blue, 50% methanol and 10% acetic acid for 20 min and destained in 5% methanol, 5% acetic acid overnight (or at 45 - 50°C for 10 - 15 min), or fixed in 7% acetic acid for 1 h at room temperature. Gels were then either stored in ddH_2O, or dried under vacuum on a piece of Whatman filterpaper in a slab gel-drier (Hoefer Scientific Instruments) for 2 h at 60 - 80°C.

3.2.4. Fluorography and autoradiography

Radiolabelled proteins separated by polyacrylamide gel electrophoresis (section 3.2.3.) were detected as follows. The proteins were fixed by staining/destaining or by using a fixing solution as described in section 3.2.3. The gel was rinsed using ddH_2O and incubated in Amplify™ (NAMP100) (Amersham Life Sciences) at room temperature for 15 - 30 min. The gel was rinsed briefly in ddH_2O, and dried under vacuum in a slab gel-drier (Hoefer Scientific Instruments) for 2 h at 70°C. The dried gel was exposed to X-ray film (Konica Medical Film AX) at -70°C and the X-ray film developed by agitating in developing solution (Ilford Multigrade Paper Developer) for 3 min or until image became visible, rinsing in H_2O, and fixing for 1 min in fixing solution (Ilfospeed Multigrade Paper Fixer). Finally, the gel was rinsed in H_2O, and allowed to dry.

3.2.5. Construction of a recombinant baculovirus for protein expression

The BAC-TO-BAC™ Baculovirus Expression System (GIBCO BRL Life Technologies) was used to construct a recombinant baculovirus for protein expression.

Insect cell culture handling techniques were essentially as described in the instruction manual provided for the BAC-TO-BAC™ Baculovirus Expression System (GIBCO BRL Life Technologies) with additional information obtained from King and Possee (1992). *Spodoptera frugiperda* (*Sf*9) cells (Highveld Biological) were grown as suspension cultures in spinner flasks at 27°C in Grace's insect cell culture medium containing 3.3 g/l yeastolate and 3.3 g/l lactalbumin hydrolysate (Highveld Biological), modified by the addition of pluronic (0.1% PLURONIC F-68 solution) (Sigma Cell Culture), containing 10% foetal calf serum (FCS) (Highveld Biological) and antibiotics. For suspension cultures the cell density was determined using a haemocytometer; trypan blue (0.4% in PBS) was added to check cell viability. Cultures were seeded at an initial density of 0.2 - 0.5 x 10^6 cells/ml, and subcultured when they reached 1 - 2 x 10^6 cells/ml.

3.2.5.1. Preparation of the donor plasmid

The donor plasmid, pFASTBAC1 expression vector, contained the polyhedrin promoter followed by an extensive MCS. The gene of interest was cloned into the donor vector in the correct orientation with respect to the polyhedrin promoter; i.e. the 5' end of the gene was inserted into the first selected site of the MCS. The integrity of recombinant clones was confirmed by automated sequencing (section 2.2.12.) before being used to transform competent DH10BAC cells.

3.2.5.2. Preparation of competent DH10BAC cells

MAX EFFICIENCY DH10BAC™ cells (GIBCO BRL Life Technologies) are provided as one of the components of the Baculovirus Expression System. If competent DH10BAC cells are not available, they can be prepared using the DMSO method (Chung and Miller, 1988).

Five ml of the *E. coli* strain was grown overnight in LB-broth at 37°C with shaking, and 1 ml of this overnight culture was used to inoculate 100 ml LB-broth. The cells were grown to early log phase (OD_{600} = 0.3 - 0.6) and

collected by centrifugation (2 860 x g for 5 min) at 4°C. The cells were resuspended in 1/10th of the culture volume TSB (10% (w/v) PEG, 5% (v/v) DMSO, 10 mM $MgCl_2$, 10 mM $MgSO_4$, in LB-broth) and incubated for 10 - 20 min on ice. Cells were used directly or were frozen away at -70°C in 400 µl aliquots containing 15% glycerol.

3.2.5.3. Transformation of competent DH10BAC cells with pFASTBAC donor plasmid

Competent DH10BAC cells (200 µl) were mixed with up to 100 ng of recombinant pFASTBAC1 donor plasmid DNA and incubated on ice for 30 min. The cells were then heat shocked at 42°C for 45 sec and chilled on ice for 2 min. This was followed by the addition of 0.9 ml TSBG (TSB plus 20mM glucose) and incubation with agitation at 37°C for 4 h before plating out 200 µl per LB-agar plate. The LB-agar plates contained 50 µg/ml kanamycin sulphate, 10 µg/ml tetracycline and 7 µg/ml gentamycin, as well as 50 µl 2% X-gal substrate and 10 µl 100 mM IPTG inducer which were added to each plate before plating. The plates were incubated at 37°C for two days.

3.2.5.4. Isolation of recombinant bacmid DNA

The following protocol, as provided by GIBCO BRL Life technologies, was adapted for the isolation of high molecular weight bacmid DNA (Luckow *et al.*, 1993), and it involved the following. Two to five ml LB-medium (containing 50 µg/ml kanamycin, 7 µg/ml gentamycin, 10 µg/ml tetracycline) was inoculated with each of the selected colonies and incubated for 16 h with shaking at 37°C. When well grown, 1.5 ml culture was transferred to an eppendorf tube and centrifuged at maximum speed (14 000 x g) in a microfuge for 1 min. The supernatant was removed, centrifugation repeated and the remaining supernatant removed. The cells were resuspended in 0.3 ml of a solution containing 15 mM Tris-HCl, pH 8 and 10 mM EDTA, after which .0.3 ml of a solution containing 0.2 N NaOH and 1% SDS was added and the sample mixed. The sample was incubated at room temperature for 5 min, after which 0.3 ml 2.5 M KAc, pH 5.5 was added, the sample mixed and left on ice for 5 - 10 min. The sample was centrifuged for 10 min at 14 000 x g. A new microfuge tube was labelled and 0.8 ml isopropanol added to it. The supernatant was transferred to the tube containing isopropanol, mixed and placed on ice for 5 - 10 min. The sample was stored overnight at -20°C or carried on directly and centrifuged for 15 min at 14 000 x g. The supernatant was removed, 0.5 ml 70% ethanol added and the sample centrifuged for 5 min at 14 000 x g. If desired, this wash step was repeated. The supernatant was removed, the pellet briefly air-dried for 5 - 10 min at room temperature and resuspended in 30 µl ddH_2O. The DNA was stored at -20°C until use.

3.2.5.5. PCR analysis of bacmid DNA to confirm the presence of the gene of interest

A standard PCR reaction was performed and the reaction contained 1.5 µl bacmid DNA, 5 µl thermophilic DNA polymerase 10 x buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl and 0.1% Triton® X-100) (Promega), 3 µl 2.5 mM dNTP mix (TaKaRa Biomedicals), 2.5 µl of each primer (POLH and M13-RP, each 100pmol/µl) (refer to Table 2.5.), 3 µl 25 mM $MgCl_2$, 0.5 µl Taq polymerase (5U/µl) (Promega), and ddH_2O to a final volume of 50 µl. The PCR program used consisted of one cycle of 2 min at 94°C, 30 cycles of 45 sec at 94°C, 45 sec at 55°C and 5 min at 72°C, followed by one cycle of 7 min at 72°C. PCR was performed using a GeneAmp PCR System 9600 (Perkin Elmer). A small volume of each reaction was analysed by 1% agarose gel electrophoresis (section

2.2.3.) using suitable DNA molecular weight size markers to estimate the size of the PCR products, and the remainder of the reactions was stored at -20°C until use.

3.2.5.6. Transfection of recombinant bacmid DNA into *Spodoptera frugiperda* (*Sf*9) cells

Cells were seeded (1 x 10^6 cells/cm^2 for 6-well plates (Nunclon™)) in Grace's medium (Highveld Biological) without supplements (antibiotics, pluronic and FCS) and allowed to attach at 27°C for at least an h. Two solutions were prepared, solution A contained 5 µl bacmid miniprep plasmid DNA diluted into 100 µl Grace's medium without supplements, and solution B contained 6 µl CELLFECTIN™ reagent (GIBCO BRL Life Technologies) diluted into 100 µl of Grace's medium without supplements. The two solutions were combined, mixed and incubated at room temperature for 45 min. The cells were washed once or twice with 2 ml Grace's medium without supplements. Grace's medium without supplements (800 µl) were added to each tube containing DNA-lipid complexes and mixed gently. The wash media were removed from the cells and the washed cells overlayed with 1 ml of the diluted DNA-lipid complexes. The cells were incubated at 27°C for 5 h. The transfection mixture was removed, 2 ml Grace's medium containing antibiotics, pluronic and FCS added, and the cells incubated at 27°C for a further 96 h.

3.2.5.7. Harvest and storage of recombinant and wild type baculoviruses

Baculoviruses were harvested from transfection or p.i. supernatants by transfer of the supernatant to a sterile, capped tube. If so desired, the supernatant could be clarified through the use of centrifugation (5 min at 500 x g). The baculovirus was then contained in the supernatant, which was again transferred to a fresh, sterile tube. The baculovirus stock was stored at 4°C, protected from light. For long term storage of baculovirus at -70°C, fetal bovine serum was added to a final concentration of 2%.

3.2.6. Infection of insect cells with a baculovirus to analyse protein expression

Analysis of recombinant or wild type baculovirus expression was carried out in 24-well plates using virus stocks harvested 96 h p.i. (section 3.2.5.7.). The protocol used was as provided by GIBCO BRL Life Technologies, and was adapted from a protocol described by Luckow and Summers (1988).

Cells were seeded (0.4 x 10^6 cells/cm^2 in 24-well plates) in Grace's medium (Highveld Biological) containing antibiotics, pluronic and FCS, and allowed to attach for at least 60 min at 27°C before washing the cells once with Grace's medium containing antibiotics, pluronic and FCS. New medium was added to each well and the appropriate volume of recombinant or wild type baculovirus stock so that the total volume per well was 500 µl. The plates were then incubated at 27°C for 72 h. The cells were harvested by removing the medium from each well and using it to rinse the cells from the well. The medium containing the cells, was put into an Eppendorf tube and the cells collected by centrifugation at 800 x g for 5 min. The supernatant was discarded and the remaining cells washed from the wells with 500 µl 1 x PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.2H_2O$, 1.4 mM KH_2PO_4, pH 7.3), which was added to the cell pellet and the cells resuspended. Again,

the cells were collected by centrifugation at 800 x g for 5 min. The cell pellet was resuspended in 40 µl 1 x PBS and stored at -20°C until use. Protein expression was analyzed by SDS-PAGE (section 3.2.3.).

3.2.7. Amplification of recombinant baculovirus and wild type baculovirus stocks

Cells were seeded (1 x 10^6 cells/cm^2 for 6-well plates) in Grace's medium (Highveld Biological) with antibiotics, pluronic and FCS, and allowed to attach at 27°C for at least an h. The media were removed from the cells and 1.8 ml medium containing antibiotics, pluronic and FCS added per well, followed by 50 - 100 µl of the virus stock that needed to be amplified. The cells were incubated at 27°C for 48 h. After 48 h, the virus was harvested as described in section 3.2.5.7.

3.2.8. ^{35}S-methionine labelling of proteins

Baculovirus-infected insect cells can be labeled with ^{35}S-methionine in methionine deficient media to monitor protein expression (Luckow and Summers, 1988).

Cells were seeded (1 x 10^6 cells/cm^2 for 6-well plates) and incubated at 27°C for at least 1 h. New Grace's medium (Highveld Biological) containing antibiotics, pluronic and FCS, as well as the appropriate volume of recombinant baculovirus stock, were added to each well so that the total volume per well was 1 ml. After incubation for 1 h at 27°C, 2 ml Grace's medium containing antibiotics, pluronic and FCS was added to each well, and the cells were incubated for 18 - 24 h at 27°C. After incubation, the medium was removed and the cells washed twice with 2 ml methionine-free Eagle's medium (minimum essential medium without L-methionine with non-essential amino acids, with L-glutamine and 2g/ml $NaHCO_3$) (Highveld Biological). The wash medium was replaced with 1 ml fresh methionine-free Eagle's medium and the cells starved for 1 h at 27°C to deplete the intracellular pools of methionine. The medium was replaced with fresh methionine-free Eagle's medium (1 ml per well) containing 30 µCi [^{35}S] methionine/ml (EASYTAG™ METHIONINE L – [^{35}S] (NEW™ Boston, MA, USA) and labelling was allowed for at least 3 h. The cells were harvested as described in section 3.2.6. and protein expression was analysed using SDS-PAGE (section 3.2.3) followed by fluorography and autoradiography (section 3.2.4.)

3.3. RESULTS

In order to determine if EEV segment 10 expressed two related proteins, as has been observed for segment 10 of other orbiviruses such as BTV, EHDV, Palyam virus, and AHSV, *in vitro* translation experiments using purified dsRNA were performed and the protein products of the NS3 gene of EEV and the other orbiviruses compared. In addition, to study the expression of EEV NS3 and to establish a system to express NS3 of EEV in sufficient quantities for future functional studies, a recombinant baculovirus was constructed to express the EEV Bryanston (EEV-1) (S1REF*) NS3 gene in the BAC-TO-BAC™ Baculovirus Expression System. The results obtained using *in vitro* translations were also compared to the expression of the EEV NS3 gene in the baculovirus expression system.

3.3.1. *In vitro* translation of dsRNA of EEV Bryanston (EEV-1) (S1REF) and other orbiviruses

In vitro translation reactions were performed using a commercially available kit of the rabbit reticulocyte lysate system (Amersham Pharmacia Biotech) according to the manufacturer's instructions (section 3.2.2.). The proteins in the translation reaction mixtures were analysed by 12% SDS-PAGE (section 3.2.4.), followed by fluorography and autoradiography (section 3.2.4.) and the results are shown in Fig. 3.1. and 3.2.

At least 10 proteins were synthesized during the *in vitro* translation reaction of dsRNA of EEV Bryanston (EEV-1) (S1REF) (Fig. 3.1.). The proteins were named using the nomenclature described for other orbiviruses by comparing the obtained EEV protein profile with that of other orbiviruses such as BTV (refer to Table 1.6.). At this stage the coding assignment of EEV is unpublished and the proteins as they are observed in Fig. 3.1. and the assignments they have been given, have not been verified, but are based only on analogy to BTV and AHSV proteins.

The *in vitro* translation experiment using dsRNA of EEV was repeated, and dsRNA of AHSV, BTV and EHDV isolates were included in order to compare the *in vitro* translation of these orbiviruses to that of EEV, specifically regarding genome segment 10. The results can be seen in Fig. 3.2. The *in vitro* translation reactions were not equally successful, e.g. the lanes containing the BTV or EHDV samples, this could be due to a variety of factors, the most important factor probably relating to the concentration of denatured dsRNA within the reaction. When the concentration of ssRNA is low, fewer templates are available for translation, which in turn leads to less protein being made. Generally, the smaller RNA segments are translated more frequently in the *in vitro* system than the larger RNA segments, which would explain the fact that the NS3 protein was observed even when all the other proteins were not.

The sizes of the NS3 proteins seen on the 12% SDS-polyacrylamide gels (Fig. 3.1. and 3.2.) were estimated using the Rainbow™ protein molecular weight marker (Amersham) as a standard and plotting $\log_{10}$ of molecular weight as a function of the distance migrated by the various proteins. The estimated size of AHSV, BTV and EHDV NS3 was approximately 24 K; the estimated size of EEV NS3 was approximately 27 K, which is similar to the predicted molecular weight (section 2.3.3.).

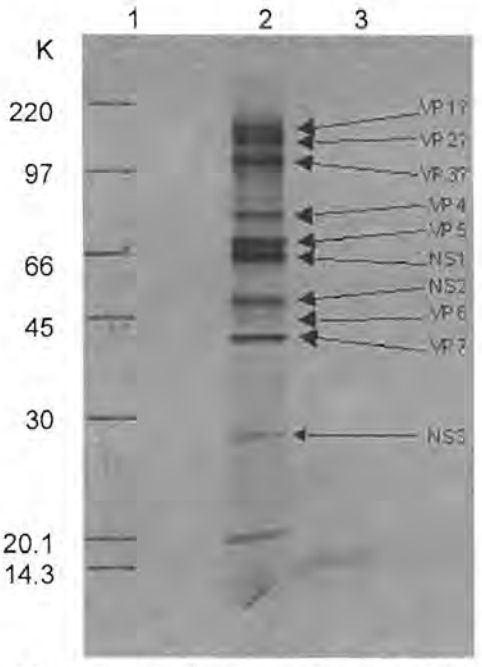

Figure 3.1. Proteins synthesized from the *in vitro* translation of dsRNA of EEV Bryanston (EEV-1) (S1REF) (2) analysed by 12% SDS-PAGE followed by fluorography and autoradiography. A negative control sample, in which no RNA was added to the *in vitro* translation reaction (3), was also included. Molecular weight standards (1) are Rainbow protein markers (non-radioactive, positions redrawn from gel). The arrows indicate the positions of the different protein products. The identities of the proteins have not been verified and the assignments are based on analogy to AHSV and BTV proteins.

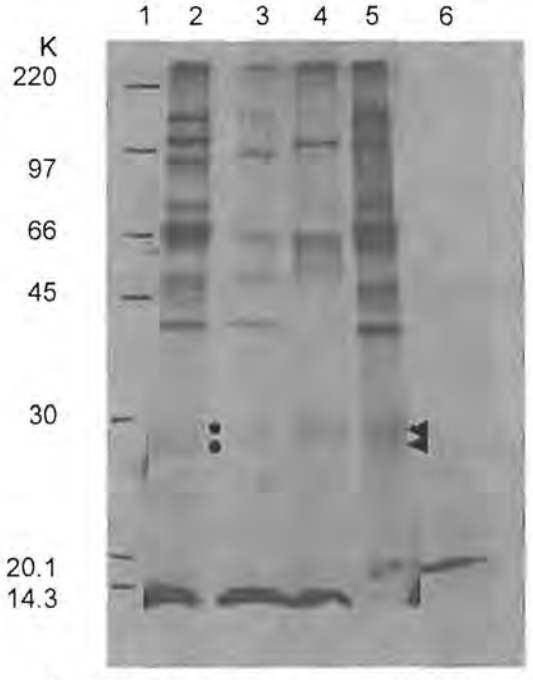

Figure 3.2. Proteins synthesized from the *in vitro* translation of dsRNA of AHSV-3 (AHSV M322/97) (3), BTV-10 (4), EHDV-1 (5) and EEV Bryanston (EEV-1) (S1REF) (6) analysed by 12% SDS-PAGE, followed by fluorography and autoradiography. A negative control sample, in which no RNA was added to the *in vitro* translation reaction (6), was also included. Molecular weight standards (1) are Rainbow protein markers (non-radioactive, positions redrawn from gel). The arrows indicate the position of NS3 and NS3A of EEV Bryanston (EEV-1) (SREF), and dots indicate NS3 and NS3A of AHSV-3 (AHSV M322/97).

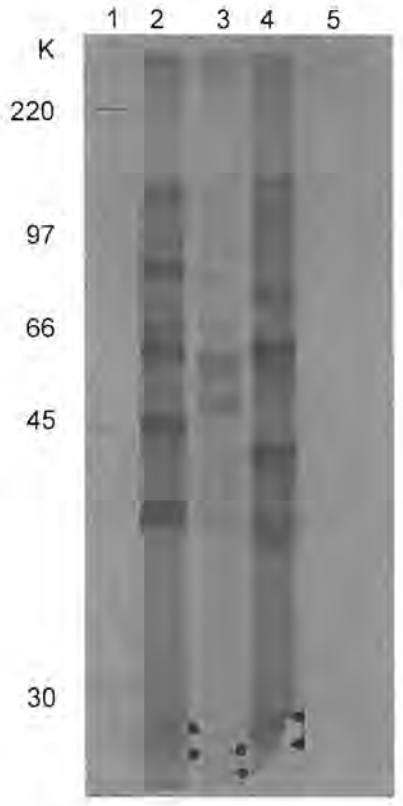

Figure 3.3. Proteins synthesized from the *in vitro* translation of dsRNA of BTV-10 (2), EHDV-1 (3) and EEV Bryanston (EEV-1) (S1REF) (4) analysed by 12% SDS-PAGE followed by fluorography and autoradiography. A negative control sample, in which no RNA was added to the *in vitro* translation reaction (5), was also included. Molecular weight standards (1) are Rainbow protein markers (non-radioactive, positions redrawn from gel). The arrows indicate the position of NS3 and NS3A of EEV Bryanston (EEV-1) (S1REF) and the dots indicate NS3 and NS3A of BTV-10 and EHDV-1.

As can be seen from the results in Fig. 3.1. and Fig. 3.2., it is not possible to distinguish NS3 and NS3A clearly. In Fig. 3.1. only one band is clearly visible, while in Fig. 3.2. the same band is fuzzy and seems to consist of two individual bands. The poor resolution can probably be attributed to the fact that small 12% gels were used that were run for a few hours at high voltage. This prevented the proper separation of the NS3 and NS3A proteins that are closely related to each other regarding their size; NS3 is estimated to be approximately 27 K, while NS3A is estimated to be approximately 25 K (refer to Table 2.6.). For this reason, the same *in vitro* translation samples used in the previous experiments (Fig. 3.1. and 3.2.) were again analysed. First, by performing 12% SDS-PAGE, allowing the gel to run for a longer time until the 20.1 K and 14.3 K markers ran off the gel (Fig.3.3.), or secondly by performing 15% SDS-PAGE overnight at a low voltage (refer to Fig.3.4.).

In Fig. 3.3., a broad fuzzy band can be seen in the region below the 30 K marker for lanes containing BTV, EHDV and EEV samples. This band seems to consist of two individual bands which are identified as NS3 and NS3A (also refer to Fig. 3.4., section 3.3.2., here two bands are again seen and their sizes estimated).

The results obtained in this section seem to indicate that EEV S10 encodes two proteins, NS3 and NS3A, as has been seen for other orbiviruses such as AHSV, BTV and EHDV. The two proteins are also produced in approximately equal amounts as can be estimated from the intensity of the bands representing the two proteins on the various autoradiographs. This result confirms the expectation created by the two in-phase initiation codons observed for EEV S10 (section 2.3.5.), namely that two proteins are encoded by S10 of EEV. The *in vitro* translation system however useful has the disadvantage that it is not a truly accurate representation of the expression of a protein or proteins in living cells. For this reason EEV S10 was also expressed using the baculovirus expression system.

3.3.2. Construction of a recombinant baculovirus for expression of EEV Bryanston (EEV-1) (S1REF*) NS3 and the expression of this protein in insect cells

The BAC-TO-BAC™ Baculovirus Expression System (GIBCO BRL Life Technologies) makes use of a baculovirus (an insect virus) which infects cultured insect cells. A rapid and efficient method has been developed by Luckow and co-workers (1993) to generate recombinant baculoviruses containing the gene of interest under the transcriptional control of the strong polyhedrin promoter of the *Autographa californica* nuclear polyhedrosis virus (AcNPV). This method is based on site-specific transposition of an expression cassette into a baculovirus shuttle vector (bacmid) which is propagated in *E. coli*.

Genome segment 10 of EEV Bryanston (EEV-1) (S1REF*) that encodes NS3, was cloned into the donor plasmid, pFASTBAC1 (section 3.2.5.1.), and the recombinant plasmid FB.EEVB.NS3 (pFASTBAC1 containing S10 of EEV Bryanston (EEV-1) (S1REF*)) was used to transform competent DH10BAC cells (sections 3.2.5.2. and 3.2.6.3). After transformation, colonies were selected on the basis of their antibiotic resistance (pFASTBAC1 confers gentamycin resistance to the cells) and colour (white colonies contain recombinant bacmid DNA that has undergone transposition). Bacmid DNA was isolated (section 3.2.5.4.) from selected colonies and was analysed by PCR (section 3.2.5.5.) to ascertain if the correct size insert had been transposed into the bacmid genome.

The PCR analysis (section 3.2.5.5.) is based on the observation that a reaction containing non-recombinant or wild type bacmid DNA generates a PCR product of approximately 723 bp in size. A reaction containing recombinant bacmid DNA should thus generate a PCR product of 723 bp plus the size of the gene of interest, in this case 759 bp, which produces a PCR product of approximately 1482 bp. PCR analysis was performed for a number of selected colonies (results not shown). In most cases, PCR products were obtained that conformed to the expected size of approximately 1482 bp and a single bacmid DNA sample was used to transfect insect cells (section 3.2.5.6.) After transfection, the putative recombinant baculovirus was harvested (section 3.2.5.7.) and stored at 4°C until use, or subjected to a round of amplification (section 3.2.7.) in order to increase the viral titre, and then stored at 4°C until use.

The next step was to test the expression of the putative recombinant baculovirus by infecting *Sf*9 cells with the virus, harvesting the virus-infected cells and performing SDS-PAGE analysis (section 3.2.7.) to determine if the EEV NS3 protein was being expressed. A number of these infection experiments (section 3.2.6.) were performed using different experimental conditions such as using different amounts of harvested putative recombinant baculovirus, using different amounts of harvested putative recombinant baculovirus after a round of amplification (section 3.2.8.), and harvesting the infected cells at different time points (24, 48, 72, and 96 hours) post infection. In each case, when comparing the cells infected with the putative recombinant baculovirus to wild type infected or mock infected cells, no unique protein band representing EEV NS3 was seen on the SDS-PAGE gels after Coomassie staining (results not shown).

The fact that no expressed NS3 protein bands were observed using Coomassie blue staining did not necessarily mean that EEV NS3 was not being expressed, but possibly that the detection technique used was not suitable or sensitive enough to detect expression; this would be the case if EEV NS3 was expressed at low levels. It was thus decided to test expression of the recombinant baculovirus using a more sensitive detection method such as labelling with radioactivity which would allow the incorporation of a radioisotope into proteins (cellular and viral) as they are synthesized in the insect cells.

Labelling of proteins with ^{35}S-methionine was performed as described in section 3.2.8. The labelling experiment was first performed using a time point of 18 hours p.i., no unique protein bands were however observed when comparing the proteins from recombinant baculovirus infected cells to the cellular proteins or the proteins generated by a wild type baculovirus infection (results not shown). The labelling experiment was repeated; the time period post infection was however increased to 24 hours, and labelling was allowed to take place for approximately 46 hours. The result of this experiment is shown in Fig. 3.4.

In Fig. 3.4. a single unique band, designated as NS3, is observed in all the samples of a recombinant baculovirus expressing EEV Bryanston (EEV-1) (S1REF*) NS3. In contrast, two bands, designated as NS3 and NS3A, are observed for the sample of an *in vitro* translation sample of an EEV Bryanston (EEV-1) field isolate (S1FLD3). This result confirms that in the baculovirus expression system, only NS3 is observed, while for *in vitro* translation reactions both NS3 and NS3A are observed. The size of the EEV Bryanston (EEV-1) (S1REF*) NS3 proteins seen on the 15% SDS-polyacrylamide gels (Fig.3.4.) were estimated as before by using the Rainbow molecular marker as a standard and plotting $\log_{10}$ of molecular weight as a function of the distance migrated by the various proteins (refer to section 3.3.1.).

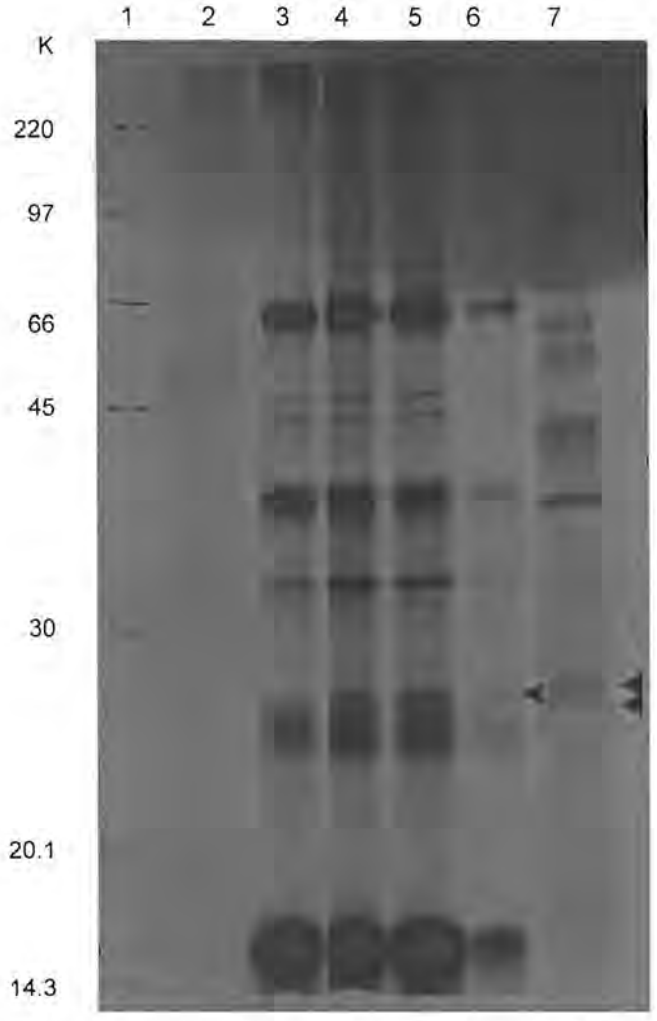

Figure 3.4. Labelling with ^{35}S-methionine of proteins synthesized in *Sf*9 cells using the baculovirus expression system. Recombinant baculovirus expressing EEV Bryanston (EEV-1) (S1REF*) NS3 (4, 5 and 6) analysed by 15% SDS-PAGE, fluorography and autoradiography. Three controls, mock infected cells (2), cells infected with wild type baculovirus (3) and an aliquot of an *in vitro* translation sample of an EEV Bryanston (EEV-1) field isolate (S1FLD3) (7), were also included. Molecular weight standards (1) are Rainbow protein markers (non-radioactive, positions redrawn from gel). The arrows indicate the position of NS3 and NS3A of EEV.

The estimated size of the single NS3 band observed in Fig.3.4. (lane 4, 5 and 6) was approximately 27 K. The sizes of the two proteins, NS3 and NS3A, seen in Fig. 3.4. (lane 7) were estimated to be approximately 28 K and 26 K respectively. The estimates for both the single NS3 band and the two bands representing NS3 and NS3A are very similar to the theoretical estimate of 27 K for NS3 and 25 K for NS3A (section 2.3.3).

Another method to test for expression of NS3 would be through the use of EEV antiserum, preferably a monospecific antiserum directed at NS3, in a Western blot procedure. An honour's project was launched in 2000 to obtain serum directed at EEV Bryanston (EEV-1) (S1REF*) NS3, but unfortunately due to technical difficulties, the project failed and no serum was obtained and made available. As will be mentioned in Chapter 4, the preparation of serum directed against EEV NS3 is

one of the priorities for future study of EEV NS3. The availability of antiserum will allow further studies of the nature of EEV NS3 expression, specifically its subcellular localization as well as variation as indicated by cross-reactivity of antiserum directed at one serotype with NS3 of other serotypes.

3.4. DISCUSSION

The aim of this part of the study was firstly to determine if both of the in-phase initiation codons identified in EEV NS3 (section 2.3.5.1.) are used when the denatured NS3 dsRNA gene is translated in an *in vitro* rabbit reticulocyte system, and secondly to construct a recombinant baculovirus that expresses NS3 of EEV. The successful expression of EEV NS3 in insect cells using the baculovirus expression system would also allow the establishment of a system to express NS3 of EEV to make future functional studies possible.

In the *in vitro* translation experiments performed using the rabbit reticulocyte lysate system, and dsRNA from EEV Bryanston (EEV-1) (S1REF) and other orbiviruses (AHSV, BTV and EHDV), ten protein bands were identified for EEV Bryanston (EEV-1) (S1REF) that were thought to represent the 7 structural proteins and the 3 non-structural proteins. The identities of the proteins were assigned using known data for other orbiviruses as a guide, since no coding assignment has yet been published for EEV; although a recent study indicates that the assignment is similar to that of AHSV and BTV (Potgieter *et al.*, 2002) (refer to section 1.4.3.). The question thus arises of which genome segment encodes which protein(s) when using all the dsRNA segments in *in vitro* translation experiments. Isolating the individual dsRNA segments before *in vitro* translation could solve this problem, however, in the case of genome segment 10, as it is the smallest genome segment, it is expected to encode the smallest protein(s). The focus on S10 in this investigation thus eliminates the need to isolate the individual dsRNA genome segments. EEV NS3/NS3A was therefore identified using both the theoretical molecular weight estimate of 27 K (section 2.3.3.) and the known molecular weights of other orbivirus NS3 proteins as guidelines (refer to Table 2.15.). In the case of EEV NS3 and NS3A, the two proteins were synthesized in approximately equal amounts, and could only be separated by means of 15% SDS-PAGE analysis. The sizes of EEV NS3 and NS3A were estimated to be approximately 28 K and 26 K respectively, which falls in the same size range as the theoretical estimate of 27 K for NS3 and 25 K for NS3A (section 2.3.3., Fig. 3.4.).

The molecular weight of the EEV Bryanston (EEV-1) (S1REF*) baculovirus expressed NS3 protein was estimated to be approximately 27 K, an estimate that again is similar to the theoretical estimate. Size differences for the same proteins can also be observed on SDS-PAGE gels in some cases. A factor to take into account when estimating molecular weight, specifically in eukaryotic expression systems such as the baculovirus system, are post-translational modifications.

Glycosylation of BTV NS3/NS3A was first detected by Wu *et al.* (1992). It has been shown that heavily glycosylated proteins can be difficult to detect, even when synthesized efficiently, as the protein can appear as a diffuse rather than a tight band on gels. Also, the glycosylation of a protein can vary, giving rise to multiple bands on a gel or to differently sized forms of the same protein under different circumstances. When NS3/NS3A proteins are glycosylated, their migration patterns on a polyacrylamide gel can therefore change giving rise to differently sized bands on a gel, which could also explain the variation seen when estimating the size of proteins. This situation is especially important when the proteins isolated were *in vivo* expressed in mammalian cells, since the glycosylation of proteins are generally more efficient in mammalian cells than in insect or yeast cells (Guirakhoo *et al.*, 1995). King and Possee (1992) has also made the observation that the glycosylation of proteins in insect cells are different from that seen in mammalian cells, this can also cause diffuse bands of varying molecular weight. The recombinant proteins in insect cells can also have a smaller apparent molecular weight, after analysis by PAGE, than their authentic counterparts in the normal host cell. In the case of EEV Bryanston (EEV-1) (S1REF*) NS3, the protein has two potential N-linked glycosylation sites (section 2.3.3.), this could potentially lead to different molecular weight estimations for NS3, especially in cases where a single diffuse band is visible on gels.

The expression of EEV NS3 in the baculovirus expression system could not be detected using Coomassie blue staining, indicating that EEV NS3 was probably expressed at low levels. Low levels of EEV NS3 expression were however not unexpected. Although large amounts of NS3 has been observed for BTV NS3 expressed in the baculovirus expression system (French *et al.*, 1989), low levels of expression has been seen for AHSV NS3 in the same expression system (Van Staden *et al.*, 1995) and the expression of AHSV NS3 was not sufficient to be detected by Coomassie blue staining (Van Niekerk *et al.*, 2001a) as was the case in this investigation for EEV NS3. EEV and AHSV have similar hosts; EEV NS3 and AHSV NS3 also share a number of structural characteristics and protein motifs (Chapter 2), it could therefore be postulated that the expression of NS3 of EEV could be more similar to that of AHSV than that of BTV and that low levels of EEV NS3 could be expected when expressed in the baculovirus expression system. In order to detect the proposed low level of expression of EEV NS3 it was decided to radiolabel the synthesized proteins with ^{35}S-methionine.

A few problems were experienced with the radiolabelling procedure (section 3.2.8.), which were most probably due to a low viral titre of the recombinant baculovirus as it is known that if the infectivity of a viral stock is too low, the insect cells will be inadequately infected and virus gene expression will be poor (King and Possee, 1992). When the time before labelling was increased from 18 hours to 24 hours and the labelling period increased from 3 hours to approximately 46 hours to compensate for the suspected low viral titre, expression of NS3 could be seen clearly, NS3A was however not observed (Fig. 3.4., lane 4, 5 and 6). This problem can however be avoided in further studies using this recombinant viral stock by performing virus titrations to determine the viral titre and by viral

amplification. It should also be noted that when using the baculovirus expression system, the foreign protein is placed under the control of the polyhedrin promoter, which is a very late promoter (active from about 18 to 72 hours p.i.). King and Possee (1992) suggest that when this promoter is used to drive expression of a foreign gene, protein synthesis should initially be examined at 24 or 48 hours p. i., since a rapid accumulation of material is observed between 24 and 30 hours p.i., followed by a more gradual increase over the final part of the infection cycle. Thereafter a time experiment can be performed to determine the point of maximum synthesis of the foreign protein. It has been found for AHSV that NS3 expression is maximal between 24 to 30 hours p.i. (Van Staden *et al.*, 1995; Van Niekerk *et al.*, 2001a). A similar finding has been made for BTV NS3. French *et al.* (1989) found that when using the baculovirus expression system, high level expression of BTV NS3 is observed in the insect cells when the cells are infected at a high MOI and harvested 24 to 48 hours p.i.

As already seen, EEV NS3 was expressed at low levels in the baculovirus expression system, while no EEV NS3A was observed. In contrast, equimolar levels of expression were observed for EEV NS3 and NS3A in *in vitro* translation experiments, and the level of NS3 seen exceeded that observed in the baculovirus system. There are a number of possible explanations for this observation. The secondary structure of ssRNA (mRNA) in *in vivo* expression systems may impede transcription/translation leading to low levels of the NS3/NS3A proteins. In the case of BTV, stable stem-loop structures have been identified in the S10 RNA sequence of BTV between the first and second AUG translation initiation codons, as well as about 40 nucleotides downstream from the second AUG triplet (Hwang *et al.*, 1992). The initiation codon for BTV NS3A might therefore be flanked by two stable stem-loop structures and this might explain the existence of disproportional amounts of NS3 and NS3A proteins found in BTV-infected cells. However, in AHSV-infected Vero cells, both NS3 and NS3A were shown to be synthesized in approximately equimolar amounts (Van Staden *et al.*, 1995) and a possible explanation for the difference in translation efficiency could again be on the level of secondary structure. AHSV S10 lacks similar structures to the two stable stem-loop structures formed on either side of the BTV NS3A initiation codon, and this could result in a similar frequency of initiation at either of the two AUGs, with equimolar amounts of NS3 and NS3A being synthesized. The use of denatured dsRNA segment 10 transcripts in *in vitro* translation experiments therefore prevents the problem of secondary structure and equal amounts of NS3 and NS3A are produced. It has however also been observed that the AHSV-3 NS3 gene contains two G+C rich sequences at its 3' non-coding terminus at nucleotide position 688 to 712 and 737 to 758 (Van Staden and Huismans, 1991; Van Staden *et al.*, 1995). BTV-1 S10 also contains similar sequences (Gould, 1988). These sequences have also been proposed to impede RNA transcription (Gould *et al.*, 1988; Van Staden and Huismans, 1991) and are therefore connected with the relative paucity of the NS3 protein and its mRNA in orbivirus infected cells. Similar sequences, which could explain the low levels of EEV NS3, have however not been identified in the 3' non-coding terminus of the NS3 gene of EEV, and it begs the question of the involvement of other factors specifically relating to EEV NS3. Another

factor that could determine the levels of expression of EEV NS3 and NS3A is the context in which the two initiation codons reside (sections 2.3.5.1. and 2.4.). King and Possee (1992) concluded that the requirement for a Kozak consensus sequence in the baculovirus expression system was debatable. In relation to the AUG start codon, the normal AcMNPV polyhedrin gene has an A at position -3; however, foreign gene coding sequences lacking this arrangement have been successfully expressed to high levels, while others with an ideal Kozak sequence have been poorly expressed. Low levels of expression could however also be due to other factors within the baculovirus expression system or the nature of the expressed protein (e.g. if it is cytotoxic protein or a membrane-bound glycoprotein, lower levels of expression can be expected) (King and Possee, 1992).

Mertens *et al.* (1984) observed a similar discrepancy in the amount of BTV NS3/NS3A synthesized in *in vitro* and *in vivo* systems. When performing *in vitro* translation experiments, using the rabbit reticulocyte lysate system, they found in the majority of cases that the relative amount of each protein synthesized *in vitro*, was similar to that of the analogous protein synthesized *in vivo* (BTV infected BHK 21 cells), except in the case of proteins 8 and 8A (NS3 and NS3A). They were synthesized in approximately equal amounts and in relatively large amounts *in vitro* compared to *in vivo*. This was confirmed by French *et al.* (1989), they reported for BTV that NS3 was predominant *in vivo*, but approximately equal amounts of NS3 and NS3A were observed *in vitro*. This may be a reflection of the relatively greater efficiency of the translation of a small RNA species *in vitro*, but may also indicate that some regulatory mechanism (or characteristic of the protein itself) reduces the relative level of expression of this genome segment *in vivo*. It has also been found in various studies of orbivirus transcription and translation that S10 is transcribed at less than half its predicted frequency (Huismans *et al.*, 1979; Van Dijk and Huismans, 1988). This explains the low level of expression of NS3 in cells, although EHDV NS3/NS3A is expressed at higher amounts in virus-infected cells than BTV NS3/NS3A, with NS3 being the primary product (Jensen *et al.*, 1994).

The expression of other orbivirus NS3 and NS3A proteins has also been studied using the baculovirus expression system. BTV NS3/NS3A are produced in very small amounts in BTV infected cells with NS3 as the primary product (Van Dijk and Huismans, 1988; French *et al.*, 1989). When using the baculovirus expression system, a high level of expression of BTV NS3 is observed in the insect cells and the synthesis of NS3A is substantially less than NS3, NS3A is only observed after pulse labelling. The identity of the NS3A protein was also confirmed by using anti BTV-10 serum (Van Dijk and Huismans, 1988; French *et al.*, 1989). AHSV NS3 and NS3A are expressed at similar low levels in AHSV-infected Vero cells. AHSV NS3 is however also synthesized in small amounts in baculovirus infected cells, and co-migrates with a cellular protein when visualized by Coomassie staining, but it can easily be identified by radiolabelling or an immunoblot. AHSV NS3A was not detected in recombinant baculovirus infected cells, and the NS3 protein remained cell associated and was not released into the tissue culture medium (Van Staden *et al.*, 1995). The relatively low level of

expression of AHSV NS3 in the baculovirus system suggests a possible cytotoxic effect of NS3 and this was supported by studies of the insect cells using the vital exclusion dye trypan blue. The number of cells unstained by the dye declined rapidly after 24 hours p. i., at 48 hours p.i. only 6% of the cells were still unstained, indicating an increase in cell death. This decline in the ability of cells in which the recombinant baculovirus was being expressed to exclude trypan blue, was found to follow immediately after the period of maximal NS3 mRNA and protein synthesis thereby showing the involvement of NS3 in the cellular death due to the disruption of the plasma membrane (Van Staden *et al.*, 1995). The cytotoxic effect seen for AHSV NS3 also raises the question of the involvement of NS3 in other virus attributes such as pathogenicity and virulence. O'Hara *et al.* (1998) used reassortment of S10 to study the difference between a virulent strain of AHSV and an avirulent strain, their experimental data confirmed the role of NS3 in virus release from cells and supported the hypothesis that S10/NS3 may influence virulence phenotypes by altering the timing of virus release (Martin *et al.*, 1998). NS3 of AHSV can also significantly influence vector competence and therefore its transmission (Riegler *et al.*, 2000). The two identified hydrophobic regions within the AHSV NS3 protein have been implicated in the cytotoxicity and virulence of the NS3 protein. Studies have indicated that these domains are necessary to disrupt the plasma membrane, as well as for a stable association with the membrane (Van Staden *et al.*, 1995; Van Staden *et al.*, 1998; Van Niekerk *et al.*, 2001a). It has also been suggested that the high level of variation seen within the AHSV NS3 protein has some functional significance regarding virulence. The level of variation in the NS3 protein may also influence the efficacy of dissemination (refer to section 2.4.). In the case of EEV NS3/NS3A, NS3 seems to be the predominant gene product *in vivo*, although approximately equal amounts of EEV NS3 and NS3A are observed *in vitro*. A similar role to that of AHSV NS3 can also be proposed for EEV NS3 as it shows low levels of expression in the same expression system, EEV NS3 and AHSV NS3 also share a number of structural similarities, including the two hydrophobic domains. This proposed hypothesis will however need to be tested experimentally before further conclusions about the role of EEV NS3 can be drawn.

In order to study the expression of EEV NS3, it will be necessary to have some means of expressing the NS3 protein and a way to specifically detect NS3/NS3A. The first has been achieved by constructing a recombinant baculovirus containing S10 of EEV Bryanston (EEV-1) (S1REF*); the second objective can be achieved by preparing monospecific antiserum directed against NS3. This will allow a number of different studies to be performed in the future as has been done for other orbivirus NS3 proteins. In the case of BTV the NS3 and NS3A proteins are synthesized late in the infection cycle, and then only in small amounts (Van Dijk and Huismans, 1988) and elucidation of the possible function of NS3 came from immunoelectron microscopic and immunofluorescence studies on BTV infected cells and NS3 expressed in mammalian and insect cells, using antibodies raised against NS3. From these studies it was seen that NS3 and NS3A are associated with intracellular smooth surfaced vesicles and are located on the surface of infected cells in association with BTV particles

which are in process of extrusion (Hyatt *et al.*, 1993). Monospecific polyclonal antibodies have also been raised against AHSV-3 NS3 (Van Staden *et al.*, 1995) in order to study the subcellular localization of AHSV NS3.

Antibodies directed against EEV NS3 can be utilized in a number of ways. Western blotting will allow the detection of denatured forms of the protein (as an alternative to labelling with ^{35}S-methionine and to determine beyond any doubt the identity of a novel band or bands on a gel as NS3/NS3A), immunoprecipitation will recognize native forms of the protein, while immunofluorescence and electron microscopy studies will identify the subcellular localization of NS3 in mammalian and insect cells. It is therefore abundantly clear that the study of EEV NS3 is in its infancy, many questions still have to be answered in order for our knowledge of EEV NS3 to be on par with that of other orbivirus NS3 proteins such as AHSV and BTV.

CHAPTER 4

CONCLUDING REMARKS

The long-term aim regarding all studies concerning NS3 is to elucidate the structure/function relationship of NS3 and specifically its role in viral infection and morphogenesis, with emphasis on the mechanism responsible for the release of viral particles from infected cells, as well as the possible role of NS3 in virulence and disease. Within that context, the focus of this investigation was on genome segment 10 of EEV and the resulting gene product(s) with the aim to characterize the EEV genome segment 10 and gene product(s) and to compare them to their orbivirus counterparts in an attempt to identify conserved and/or unique characteristics. In order to achieve this aim, a number of short term aims relating to EEV NS3 were investigated and the details of the results that were obtained in the course of achieving these objectives were discussed in the individual chapters. In this conclusion, the main results which could contribute to a better understanding of the molecular nature of the NS3 protein of EEV will be summarized and some suggestions regarding future research in this field will be made.

A number of conserved structural features or characteristics have been identified for EEV NS3 within the EEV serogroup. These features include two putative initiation codons, two hydrophobic domains with the potential to form transmembrane helices, a coiled-coil domain, a proline-rich region, glycosylation sites, myristylation sites, a conserved region, a variable region and conserved cysteine residues. Similar features or characteristics have been found in other orbivirus NS3 proteins such as AHSV NS3, BTV NS3 and EHDV NS3.

The hydrophobic regions suggest that the EEV NS3 protein is a membrane protein and as such the membrane topology of the protein was investigated. The membrane topology of EEV NS3 was shown to be similar to both the AHSV model and the EHDV model, not excluding either one of the models. The identified coiled-coil domain and the conserved cysteine residues could play a role in dimerization as was proposed for EHDV NS3 where NS3 protein dimers form channels or viroporins. These viroporins play a role in viral release and cell damage by disrupting the cellular membrane by changing membrane permeability and interfering with cellular ionic gradients, most likely intracellular calcium levels. EEV NS3 also contains a putative bipartite membrane targeting signal, a signal peptide and a potential cleavage site for the signal peptide. The bipartite signal consists of a conserved myristylation site and a stretch of positively charged amino acids and is found within the identified conserved region. Using the information available, it is possible to predict that EEV NS3 will probably be targeted to the ER from where it will later be transported via the Golgi complex to the correct destination, which is most probably the plasma membrane of the host cell.

The predicted membrane topology of EEV NS3 also correlated with the identified variable region. This region is located between the two hydrophobic domains and as such is predicted to be exposed extracellularly. This region is also predicted to possess antigenic properties, which could play a role in the virus-host immunological interaction. The identified proline-rich region is found in the N-terminal portion of the protein that is predicted to be located in the cytoplasm, this region could function by binding other viral proteins in order to perform a function as a receptor in analogy with rotavirus NSP4.

The variation found in the NS3 protein is not confined to the variable region and has certain useful applications, including epidemiological studies where the outbreak of disease within a region is traced to the source. The maximum variation observed within the EEV serogroup between serotypes is 25.2% on a nucleotide level and 16.7% on an amino acid level. These values indicate that EEV NS3 is more variable than BTV NS3, but less variable than AHSV NS3. This level of variability within EEV NS3 was also observed between the two subclusters (A and B) in the phylogenetic trees and indicated that EEV NS3 could be grouped into two groups according to firstly, serotype and secondly, geographic location. A similar observation has been made for BTV where 3 groups (2 USA and 1 Asian) were identified based only on geographic origin. This implies that EEV NS3 has topotyping characteristics and that based on S10 or NS3 sequences the possible origins of a viral isolate can be predicted. As already discussed, there also seems to be a link between vector distribution and the distribution of the EEV serotypes within the two regions as indicated by the grouping of EEV in the two subclusters (A and B). A question now arises regarding the evolution of the EEV serogroup. Is there evidence for a co-speciating event where a specific vector is better adapted to disseminate a specific EEV serotype in a specific region? These types of questions, as well as the specific grouping pattern within the EEV serogroup will have to be investigated by sequencing more different isolates and more recent field isolates. The phylogeny of both S10 and NS3 also indicated that the EEV serogroup was a distinct lineage separate from the other orbivirus groups. In this investigation it was also found that a single set of primers based on EEV Kyalami (EEV-5) S10 could amplify all seven known different serotypes of EEV S10 by using RT-PCR, but no amplification was observed for EEV S10 when using primers directed at AHSV S10. This provides another way, by using RT-PCR, in addition to the agarose dsRNA profiles, to distinguish between AHSV and EEV during infection.

The expression of EEV NS3/NS3A was investigated using both *in vitro* and *in vivo* systems. *In vitro* translation studies were performed using EEV dsRNA and two proteins, NS3 and NS3A, were observed that were expressed in approximately equimolar amounts. In the baculovirus expression system, a recombinant baculovirus expressing EEV S10 was constructed, and expression of one protein, NS3, was observed at low levels and only after labelling with ^{35}S-methionine. The low levels of expression in the *in vivo* system prohibit the purification of the protein in its native form, and can possibly be explained by the context in which the initiation codons are found. A comparison with the

Kozak consensus sequence of initiation indicated that the two putative initiation codons are both found in suboptimal contexts for the initiation of translation. In addition, in *in vivo* systems RNA secondary structure can play a role in impeding translation by forming hairpin loops in the vicinity of the initiation codons.

The identified conserved structural features, the variation within NS3 of the EEV serogroup and the expression of EEV NS3, all hold clues as to the role EEV NS3 will play in the viral lifecycle. Some of these potential roles have been mentioned in the preceding chapters and in the preceding paragraphs. The work done and described in this investigation will therefore form the basis of future studies of EEV NS3.

The relationship between structure and function has to be proven experimentally and in this regard there are a large number of experiments that will have to be conducted in future research projects in order to elucidate the relationship. It is important to link the features that are conserved amongst the NS3 proteins of different orbiviruses to specific functions. The study of the functional significance/importance of these domains will be important not only for the EEV serogroup but also for the *Orbivirus* genus as a whole.

Future research should involve investigations relating to the subcellular localization of EEV NS3, cytotoxicity studies which could provide a link to virulence, and further sequencing of EEV S10/NS3, including more recent field isolates, to confirm the levels of variation observed as well as the grouping within the EEV serogroup.

In order to continue with these future projects a number of molecular tools are required. Firstly, monospecific antiserum directed against EEV Bryanston (EEV-1) (S1REF*) NS3 is needed. The use of antiserum will allow the detection of expression of EEV NS3/NS3A in *in vitro* and *in vivo* systems without the use of radiolabelled amino acids by Western blotting, as well as the identification of its subcellular localization in insect and mammalian cells in immunofluorescence studies as has been done for AHSV NS3 (Stoltz *et al.*, 1996). The identification of the location of a viral protein in an infected cell can give an indication of the function of the protein and the association of a particular protein with specific cellular and viral structures or events may provide insight in its role in virus morphogenesis.

The second tool that is needed is a way to express sufficient quantities of the protein under investigation. As already mentioned, the low levels of expression of EEV NS3/NS3A in the baculovirus system prohibits the purification of sufficient quantities of these proteins for functional studies. The low levels of expression obtained in the baculovirus expression system when using *Sf*9 cells can however be increased by using an alternative cell line derived from *Mamestra brassicae*

(Mb) as it appears to provide an increase in both polyhedrin and foreign protein levels that are about two- to three-fold higher than are given with *Sf*9 cells. Unfortunately, these cells are not as easy to use as the *Sf* cell lines. The production of recombinant protein in insect cells can also be scaled up (for methods refer to King and Possee, 1992); the nature of EEV NS3 (predicted to be a membrane glycoprotein and possibly cytotoxic) could however limit the amount of protein that can be produced. Therefore, the use of a bacterial expression system will be necessary to produce a sufficient amount of the protein for the preparation of monospecific antiserum or to experimentally study and confirm the predicted structural characteristics. An interesting study to perform, for which sufficient amounts of protein are needed, would be peptide mapping to experimentally identify the second initiation codon and to confirm the NS3/NS3A relationship for EEV suggested by the amino acid sequences.

The first future research project mentioned involves cytotoxicity. As already discussed, low levels of expression of a specific protein can be linked to cytotoxicity, this has been proven in the case of AHSV NS3 (Van Staden *et al.*, 1995; Van Staden *et al.*, 1998; Van Niekerk *et al.*, 2001a). The low levels of expression of AHSV NS3 in the baculovirus system were linked to cytotoxicity and the regions involved in the cytotoxic function of AHSV NS3 were also identified namely the two hydrophobic regions. As EEV NS3 exhibits a similar low level of expression in the same system, and also contains similar structural features such as the hydrophobic regions, cytotoxicity studies should be performed for EEV NS3. The information gathered in this investigation predicts that EEV NS3 could possibly show a similar cytotoxic effect as that observed for AHSV NS3. When cytotoxicity has been shown for EEV NS3 it will also be necessary to identify the regions involved in this function. The availability of mutant forms of the EEV NS3 protein would provide the opportunity to analyse the relationship between the structure and function of the protein by enabling identification of domains or individual amino acid residues that are essential for the structural integrity and/or function of the protein. These mutant forms of the EEV NS3 protein can be obtained by using site directed mutagenesis to construct recombinant baculoviruses expressing the mutant proteins as has been done for AHSV NS3 (Van Staden *et al.*, 1998; Van Niekerk *et al.*, 2001a).

A number of structural features or protein motifs were predicted for EEV NS3, e.g. glycosylation sites and myristylation sites. These sites involve post-translational modification of the EEV NS3 protein. The analysis of these post-translational processing events can be performed in insect cells and a number of methods to do this have been described (King and Possee, 1992).

In mammalian and insect cells, treating the cells with tunicamycin may inhibit N-linked glycosylation; this will allow the researcher to determine if EEV NS3 is glycosylated. Comparison of mammalian cell- and insect cell-derived glycoproteins, however, has revealed several differences in the nature of the added oligosaccharide side chains. However, where assays of biological activity have been carried out, glycoproteins synthesized in insect cells have been shown to have similar, if not identical,

activities to their authentic counterparts synthesized in mammalian cells and in most cases the glycoproteins synthesized were antigenic, giving rise to high-titre antibody preparations after injection into animals. Glycosylation in insect cells, however, might be slightly less efficient than in mammalian cells. There are thus differences in glycosylation between insect and other cell types. Whether this has a bearing on the biological activity or antigenicity of any given glycoprotein will probably have to be tested empirically for each example. This however implies that any analysis of glycosylation should be repeated in mammalian cells. Recombinant glycoproteins synthesized in insect cells may be readily detected by radiolabelling with [^{3}H] or [^{14}C] mannose or by binding to mannose-specific lectins such as concanavalin A. In addition, the baculovirus expression system can also be used to analyse phosphorylation, palmitylation or myristylation. Transcription can also be analysed, i.e.; the amount of RNA produced by the recombinant virus, if yields of the foreign protein as assessed by protein gels are low. This type of analysis should provide insight in whether the low levels of expression are due to the RNA secondary structure (transcriptional level) or the nature of the protein (translational level).

This investigation has provided important information regarding the nature of genome segment 10 and its encoded gene product NS3 of EEV. The molecular characterization of equine encephalosis virus non-structural protein NS3 is by no means complete, a solid foundation has however been laid for future research. This investigation provides structural information, as well as information about the expression of EEV NS3, that will form the basis of future functional studies of EEV NS3.