CHAPTER 4

EXPRESSION ANALYSIS OF VP1 OF AHSV-9

1. INTRODUCTION

Advances in gene manipulations have made it possible to express foreign genes in heterologous systems. In general, the cloning and subsequent expression of individual viral proteins can assist in the elucidation of the function of each protein in viral structure, replication and assembly. Analysis of the functional and molecular properties of expressed gene products, alone or in conjunction with other viral proteins, can also allow the intrinsic properties of each gene product to be determined. In addition, the availability of high levels of individual proteins allows the functions of specific viral proteins to be more easily and directly dissected.

Various systems have been developed for heterologous expression and functional analysis of putative viral polymerases. Although bacterial expression is usually efficient and inexpensive to perform, biologically active eucaryotic proteins often require post-translational modifications that are not carried out by bacterial cells. Thus many eucaryotic proteins synthesised in bacteria fold incorrectly or inefficiently and, consequently, exhibit low specific activities. Recently, however, purified recombinant protein P2 (45kDa) of dsRNA bacteriophage ϕ6 expressed in bacterial cell culture was shown to possess specific replicase activity in *in vitro* assays (Makeyev & Bamford 2000). The enzyme efficiently utilised phage-specific, positive-sense RNA substrates to produce dsRNA molecules.

In the orbiviruses, the use of baculovirus recombinants for heterologous expression of viral polymerases has been more commonly reported. In the case of BTV, Urakawa *et al.* (1989) analysed lysates of cells infected with a recombinant baculovirus expressing BTV VP1 for extraneous RNA polymerase activity, reporting the incorporation of α^{32}P-ATP into the acid-precipitatable products upon incubation with a poly(U) template and oligo(A) primer. More recently, BTV VP1 was shown to possess BTV-specific RNA polymerase activity,

synthesising full-length negative-strand RNA on positive-strand template (Devi & Roy 1999). The activity was initiated without any primer, but required an intact 3' sequence. Protein λ3 of reoviruses has been shown to be a poly(C)-dependent poly(G) polymerase when expressed under the control of the T7 RNA polymerase promoter in a recombinant VACV (Starnes & Joklik 1993). Purified λ3 was found not to transcribe reovirus-specific dsRNA into ssRNA, nor to replicate plus-strand reovirus RNA into minus-strand RNA. Complexes formed with λ1 and λ2 were also found to be incapable of transcription. On the other hand, *in vitro* sequence-specific replication assays of baculovirus-expressed rotavirus RNA polymerase (VP1) demonstrated a prerequisite for co-representation of the polymerase and the core shell protein (VP2) for synthesis of full-length dsRNA on positive-sense template RNA, even in assays containing a primer-linked RNA template (Patton *et al.* 1997). In these experiments, native mRNA's or *in vitro* cDNA-derived transcripts, with bona fide 5' and 3' termini, were utilised as templates.

The advantages of these eucaryotic expression systems are that they maximise the likelihood of appropriate post-translational modification and folding. An alternative *in vivo* approach to assaying viral polymerase activity that has already been previously discussed in chapter 1 is also of particular technical interest. This approach utilises a VACV bacteriophage T7 DNA-dependent RNA polymerase recombinant (Fuerst *et al.* 1986) which provides T7 RNA polymerase in infected cells to drive transcription from the T7 RNA polymerase promoter of a foreign gene inserted into a transfected DNA vector. The transcript is cleaved *in vivo* through either a downstream satellite *Tobacco ringspot virus* (Dzianott & Bujarski 1988) or HDV (Perrotta & Been 1991) ribozyme sequence to yield an authentic 3' end. This strategy was used to assay for specific replicase activity of the polymerase of *Flock house virus* (FHV), a bipartite positive-sense RNA virus, by transcription of the two genomic RNA's *in vivo* (Ball 1992; Ball 1994). The larger of the two genomic RNA segments (RNA 1), which encodes the entire viral contribution of the RNA replicase, was able to direct its own replication in the absence of the smaller RNA (RNA 2). However, both RNA 1 and RNA 2 were templates for the replicase, which was shown to synthesise the corresponding negative-sense RNA's, and the RNA 1-encoded sub-genomic RNA 3 also produced in FHV-infected cells.

Considering the *in vivo* nature of the latter system, capable of providing essential cellular cofactors for transcription or replication, and the possibilities for

investigation of the role of other viral proteins in viral replication, this technology was acquired and implemented for investigation of AHSV RNA polymerase activity. In addition, the proven robustness of the baculovirus system for the investigation of orbivirus polymerases was also exploited. Finally, the simplicity of bacterial expression prompted brief investigation of this approach.

2. MATERIALS AND METHODS

2.1 *In vivo* polymerase assays

2.1.1 Cells and viruses: African green monkey kidney (BSC 40) cells (Hruby *et al.* 1979), obtained from Prof. LA Ball (UAB, Birmingham, Alabama, USA), were grown as monolayer cultures in Dulbecco's modified Eagles' medium (DMEM) containing 5% foetal calf serum (FCS). Prof. LA Ball also kindly provided the recombinant VACV (Fuerst *et al.* 1986) that expresses the bacteriophage T7 RNA polymerase (vTF7-3). The virus was maintained and titred on BSC 40 cells.

2.1.2 Vectors and plasmids: Vector V(2,0), suitable for T7 RNA polymerase-driven transcription of an inserted gene and downstream HDV ribozyme-mediated cleavage (Figure 4.1), was obtained from Prof. LA Ball (UAB, Birmingham, Alabama, USA). Plasmid V(2,0) was grown in *dcm*-negative BL21(DE3) cells for digestion with *Stu* I and *Sma* I prior to dephosphorylation and utilisation as vector for cloning of blunt-ended genes. Control plasmids (FHV1(1,0) and FHV2(0,0)), containing cDNA inserts of the FHV RNA 1 and 2 under T7 RNA polymerase promoter control and upstream of an HDV ribozyme sequence, were also kindly provided by Prof. LA Ball (Ball 1994). cDNA clones of the genes encoding AHSV-9 VP3 (Maree *et al.* 1998) and NS2 (Van Staden *et al.* 1991) were obtained from the laboratory of Prof. H Huismans (UP, Pretoria, South Africa). The AHSV-9 genome segment 3 clone lacked 26 and 43 non-coding nucleotides at the 5' and 3' termini respectively, but included additional terminal *Bgl* II sites.

2.1.3 Plasmid constructions: Two oligonucleotides, with sequences 5' GTTTATTTGAGCGATGG 3' (S1.1) and 5' GTAAGTGTTTTGAGCTG 3' (S1.2), complementary to the 5' terminal sequences of the plus- and minus-strands of AHSV-9 segment 1 respectively, were used as primers in a PCR to amplify the full-length gene from 91.pBR. 10ng template was mixed with 100pmol of each primer, 5µl 10X PCR buffer (100mM Tris-HCl pH8.85, 250 mM KCl, 50 mM $(NH_4)_2SO_4$; Boehringer Mannheim), 5µl 25mM $MgSO_4$, 5µl 2mM dNTP mix and 2.5 units Pwo DNA polymerase

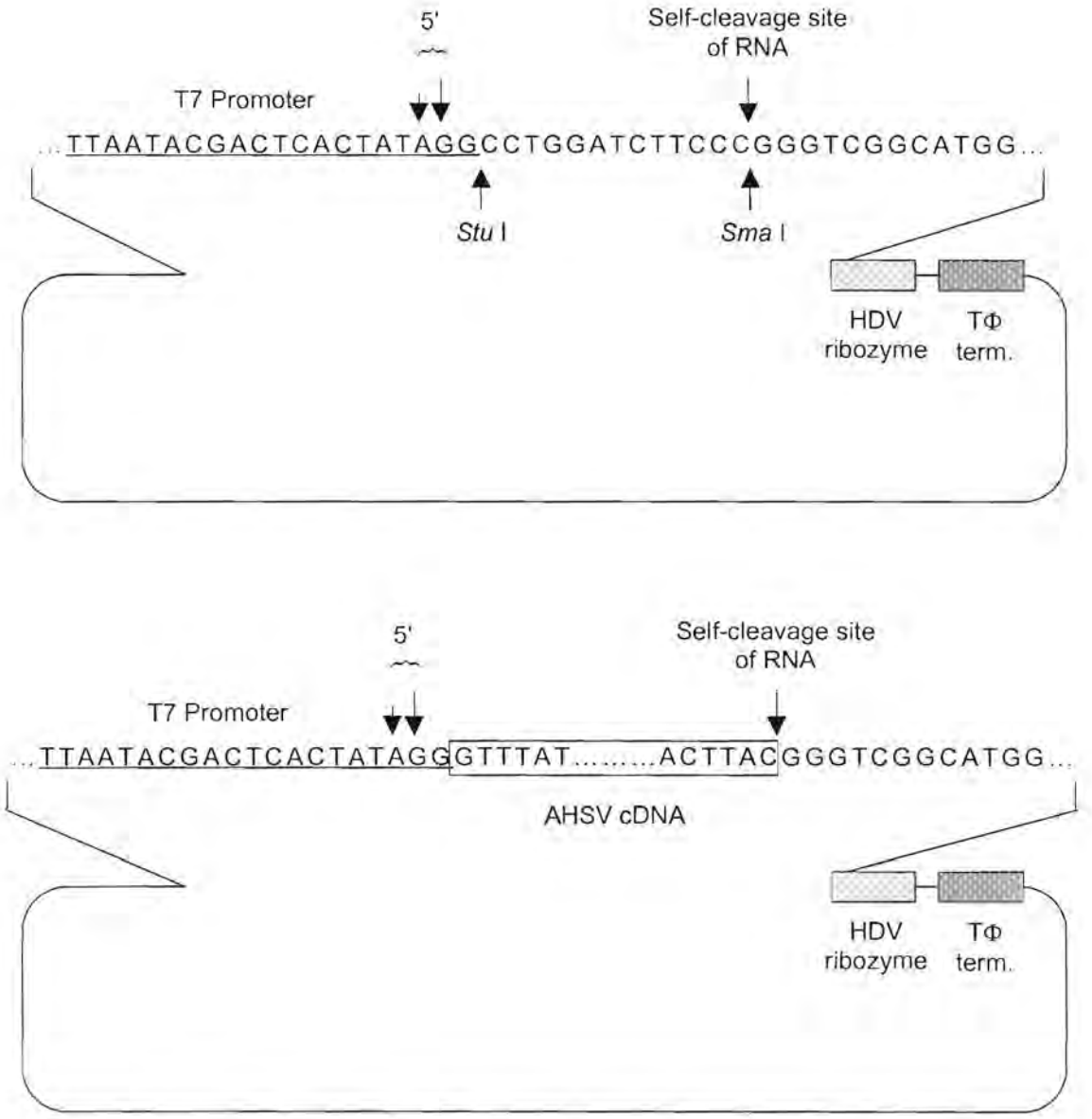

Figure 4.1 Diagrammatic representation of the structure of V(2,0) (top) and the construction of recombinant transcription plasmid (bottom) used to generate AHSV-specific transcripts. The shaded boxes represent cDNA encoding the HDV self-cleaving ribozyme and the bacteriophage T7 transcriptional terminator (TΦ term.). The *Stu* I and *Sma* I sites represent sites for foreign gene insertion, and the open box the cloned AHSV cDNA sequence. The nucleotides designated 5′ correspond to the nucleotides predicted for the 5′ end, and the self-cleavage site the predicted 3′ end, of RNA transcripts synthesized by T7 RNA polymerase.

(Boehringer Mannheim) in a final volume of 50µl. An initial cycle of 1.5 min 94°C, 45 s 47°C, 3 min 72°C was followed by 25 cycles of 30 s 94°C, 30 s 47°C, 3 min 72°C and a final cycle of 30 s 94°C, 30 s 47°C, 7 min 72°C. Amplification of a 4 kb fragment was confirmed by agarose gel electrophoresis. The amplicon was phosphorylated by incubation of 20µl of the PCR reaction mix with 20 units T4 polynucleotide kinase (PNK; Amersham) in the presence of 1mM ATP and 1X PNK buffer (70mM Tris-HCl (pH7.6), 10mM $MgCl_2$, 5mM DTT), in a final volume of 50µl. The PNK was heat inactivated at 65°C for 3 min prior to terminal polishing with 4 units DNA polymerase I Klenow fragment (Amersham) in the presence of 2µM of each dNTP for 20 min at 37°C. The amplicon was then isolated by agarose gel electrophoresis and purified by Qiaquick (Qiagen) gel extraction for ligation into blunt-ended V(2,0), to yield 91(2,0)p, 91(2,0-3)p and 91(2,0)n. Plasmid 91ΔK(2,0)p was constructed by deletion of a *Kpn* I fragment (residues 667 – 2557 of the AHSV-9 VP1 gene) from 91(2,0)p to serve as control.

An additional clone of authentic AHSV-9 segment 1 cDNA in V(2,0), with preferred adjacent sequences for efficient transcription initiation by T7 RNA polymerase, was prepared. 10ng 91.pBR was mixed with 100pmol each of S1.1 and S1.2, 10µl 10X reaction buffer (100mM Tris-HCl pH8.6, 500mM KCl, 15mM $MgCl_2$, 1% Triton X-100; Gibco BRL), 2µl 10mM dNTP mix and 2.5 units *Taq* DNA polymerase (Gibco BRL) in a final volume of 100µl. An initial cycle of 1 min 95°C, 30 s 54°C, 3 min 72°C was followed by 25 cycles of 30 s 95°C, 30 s 54°C, 3 min 72°C and a final cycle of 30 s 95°C, 30 s 54°C, 12 min 72°C. Amplification of a 4 kb fragment was confirmed by agarose gel electrophoresis. The PCR amplicon was ethanol precipitated and cloned into pGEM-T vector (Promega) according to the manufacturer's instructions, to yield 91.pGEM-T. The majority of the coding region of this gene was replaced with cDNA from 91.pBR as follows: 91.pGEM-T was fully digested with *Xho* I, in position 2980 of the VP1 gene, and partially digested with *Alw* NI, such that the sites in positions 689 and 3756 of the VP1 gene were digested, but leaving the recognition site in the pGEM-T vector undigested. The corresponding *Alw* NI fragment of the VP1 gene from 91.pBR was inserted by ligation. Similarly, the *Bgl* II fragment (nucleotides 37 to 2561 of the AHSV-9 VP1 gene) was replaced from 91.pBR, to yield 91c.pGEM-T. Sequences of the PCR-derived termini not replaced with cDNA were confirmed by sequencing. The AHSV-9 VP1 gene was subsequently isolated by *Sac* II and *Not* I digestion, blunt-ended with Mung bean nuclease and then subcloned into V(2,0), to yield 91(8,4)p.

The AHSV-9 NS2 gene was cloned into V(2,0) by PCR amplification using Expand *Taq* polymerase (Boehringer Mannheim) and blunt-ended ligation, to yield 98(2,0)p and 98(2,0)n.

The AHSV-9 VP3 gene was subcloned into V(2,0) by standard techniques involving digestion of inserted terminal restriction enzyme sites, Klenow fragment polishing and ligation, to yield 93(7-26,5-43)p and 93(7-43,5-26)n.

2.1.4 Sequencing: Junction sites and gene termini of all subclones in V(2,0) were confirmed by sequencing, using the Sequenase version 2.0 kit according to the manufacturer's instructions.

2.1.5 *In vitro* transcription and translation: Equal quantities of 91(8,4)p and 91(2,0)p were both *Bam* HI and placebo digested, followed by phenol/chloroform extraction and ethanol precipitation. Approximately 1µg of each template DNA was then mixed with 4µl 5X transcription buffer (200mM Tris-HCl pH7.9, 50mM NaCl, 30mM $MgCl_2$, 10mM spermidine; Promega), 2µl 100mM DTT, 0.5mM each rNTP, 25 units HPRI and 20 units T7 RNA polymerase (Promega) in a final volume of 20µl. Incubation at 37°C was carried out for 2 h, whereafter 2µl of the reaction mix was analysed by agarose gel electrophoresis.

In vitro translation of transcribed RNA was carried out with the wheat germ extract *in vitro* translation kit (Promega) according to the manufacturer's instructions. This involved mixing 2µl transcription reaction mix with 12.5µl wheat germ extract, 2µl 1mM amino acid mixture minus methionine, 1µl ^{35}S-methionine (10mCi/ml), 1.3µl 1M potassium acetate and 25 units HPRI in 25µl final volume. Incubation at 28°C for 90 min was followed by SDS-12% PAGE analysis of 10µl translation mix and autoradiography. Alternatively, where indicated, the rabbit reticulocyte lysate *in vitro* translation system (Promega) was used according to the manufacturer's instructions.

2.1.6 *In vivo* protein expression, labelling and analysis: Monolayers of BSC 40 cells were washed once with phosphate-buffered saline (PBS; 8g NaCl, 0.2g KCl, 2.68g Na_2HPO_4–$7H_2O$, 0.24g KH_2PO_4 per liter, pH7.4) containing 1mM $MgCl_2$ (PBSM), prior to infection with vTF7-3 at an MOI of 5 pfu per cell in PBSM. After virus adsorption at room temperature for 60 minutes, the inoculum was removed and the cells were washed once with fresh PBSM. 1ml DMEM per 35mm well was applied and the cells were incubated for 15 to 30 min at 28°C in an atmosphere of 5% CO_2. Thereafter, a mixture of 5µg CsCl- or Qiagen plasmid kit-purified DNA in 10µl TE and 10µl 1µg/µl lipofectin (Gibco BRL) was added directly to the medium. Proteins were expressed in infected and transfected cells by incubation at 28°C.

Infected and transfected cell monolayers were starved of methionine at 21 h post infection by washing twice with PBS and once with methionine-free Eagles' medium and then incubating at 28°C in methionine-free Eagles' medium for 1 h. The medium was

then replaced with 1ml methionine-free Eagles' medium supplemented with 60µCi ^{35}S-methionine per ml and incubation at 28°C was continued for 2 h. The monolayers were washed with PBS, and the cells were harvested in 300µl 1X PSB (125mM Tris-HCl pH6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol) per 35mm well. 30µl samples were analysed by standard SDS-PAGE gel electrophoresis and autoradiography.

2.1.7 RNA polymerase assay: Infected and transfected cells were incubated at 28°C for a period of 21 to 24 h. In order to inhibit DNA-dependent RNA synthesis prior to labelling the products of RNA replication, the BSC 40 cell monolayers were treated with 10µg of actinomycin D per ml for 30 min at 28°C. The medium was then replaced with 1ml per well DMEM containing 20µCi of ^{3}H-uridine per ml and 10µg actinomycin D per ml, and incubation was continued for 2 h at 28°C in 5% CO_2. Total RNA was extracted from the cells as described by the protocols using the Promega RNAgents total RNA isolation system. Cells were lysed in 500µl denaturing solution per well, with the addition of 1/10 volume 2M NaAc pH4. This was followed by a phenol/chloroform/isoamyl alcohol extraction and isopropanol precipitation. The washed and dried RNA pellets were resuspended in 20µl RNase-free water. 5µl RNA was treated with formaldehyde at 65°C in the presence of formamide and subjected to electrophoresis on 1% agarose-formaldehyde gels (Lehrach *et al.* 1977). After electrophoresis, the gels were fixed with 30% methanol - 10% acetic acid and subjected to fluorography. The latter involved equilibrating the gel in methanol, and then impregnating the gel with 3% PPO (2,5-diphenyloxazole) in methanol for 3 h. The methanol was removed by soaking the gel in water for an hour. After drying, the gel was exposed to X-ray film.

2.1.8 Northern hybridisation: Purified RNA was heat denatured at 70°C for 5 min and dot-blotted onto Hybond N+ membrane. The RNA was fixed by UV exposure for 10 min and baking at 90 – 100°C for 30 min. The blots were prehybridised overnight at 42°C in hybridisation buffer (5X SSPE (0.9M NaCl, 50mM sodium phosphate, 5mM EDTA pH7.7), 5X Denhardt's solution, 0.1% SDS, 50% formamide). ^{32}P-labelled RNA probe, synthesised by incorporation of ^{32}P-ATP during *in vitro* transcription of 91(2,0)p or 91(2,0)n, was heat denatured at 70°C for 5 min, added directly to the hybridisation bag and allowed to hybridise overnight at 42°C. The filters were washed successively in 1X SSPE, 0.1% SDS at room temperature and twice in 0.1X SSPE, 0.1% SDS at 60°C. The wet filters were autoradiographed at –70°C with intensifying screens.

2.2 Baculovirus protein expression: The BAC-TO-BAC baculovirus expression system (Gibco BRL) was utilised for expression purposes according to the manufacturer's instructions.

2.2.1 Cells: *Spodoptera frugiperda* (Sf9) cells were obtained from the American Type Culture Collection (ATCC CRL 1711). All cell culture handling techniques were essentially as described by Summers and Smith (1987). The Sf9 cells were grown as confluent monolayers or as suspension cultures in spinner flasks at 28°C in Grace's insect medium modified with antibiotic-antimycotic solution (Highveld Biological) and 10% FCS.

2.2.2 Plasmid constructions: In order to allow correctly oriented cloning of the AHSV-9 VP1 gene into the baculovirus transfer vector, the *Sph* I- and *Bam* HI-restricted gene from 91c.pGEM-T was subcloned into *Sph* I- and *Bgl* II-digested pMTL24 (Public Health Laboratory Service; 91.pMTL24). The *Bam* HI-restricted gene was then cloned into the *Bam* HI site of pFastBac1 (Gibco BRL) to yield 91.pFB.

Removal of a termination codon in the 5' non-coding region of the AHSV-9 VP1 gene to allow expression of NH_2 histidine-tagged VP1 was achieved by PCR modification of the gene in 91.pGEM3z with primers 9p1.1 (5' CGGGATCCATGGTCATCACCGTGC 3') and S1.2. 9p1.1 was complementary to the 5' terminal of the genome segment 1 open reading frame, incorporating an *Nco* I restriction enzyme recognition sequence (underlined) overlapping the VP1 gene translation initiation sequence. S1.2 was complementary to the 3' non-coding sequence of the AHSV-9 genome segment 1, without terminal extension. The PCR amplicon was Klenow polished and then *Nco* I digested to clone into *Nco* I- and *Stu* I-digested pFastBacHT (Gibco BRL) vector (yielding 91orf.pFB-HT).

2.2.3 Transposition and preparation of recombinant bacmid DNA: 91.pFB and 91orf.pFB-HT were transformed into $CaCl_2$-prepared competent DH10Bac cells (Gibco BRL) by standard techniques. Selection on kanamycin/gentamycin/tetracycline/X-gal/IPTG plates enabled identification of white colonies containing recombinant bacmid DNA. The colonies were re-streaked to confirm that they were truly white, and recombinant bacmid DNA was isolated from liquid culture by standard mini-preparations. Insertion of the VP1 gene was verified by PCR analysis of the recombinant bacmid DNA by amplification with combinations of the pUC/M13, 9p1.1 and S1.2 primers.

2.2.4 Transfection: 10µl miniprep bacmid DNA mixed with 10µl cellfectin reagent (Gibco BRL) in Grace's medium without FCS or antibiotics was transfected into monolayers of Sf9 cell in 35mm wells. The cells were incubated at 28°C for 5 h before

the transfection supernatant was replaced with 2ml Grace's medium. Virus was harvested after a further 48 h incubation period.

2.2.5 Baculovirus infection: Monolayers of 9 x 10^5 Sf9 cells per 35mm well were infected with baculovirus at an MOI of 10. Incubation was carried out for 3-4 days at 28°C.

2.2.6 Expressed protein analysis: Infected cells were harvested in 1ml 1X PBS per 35mm well, pelleted at 1000 rpm in a benchtop centrifuge, and resuspended in 30µl 1X PSB. 10µl samples were boiled for 5 minutes, sonicated for 2 minutes and analysed by SDS-8% PAGE. Following electrophoresis, gels were stained with Coomassie blue, dried and autoradiographed if applicable.

2.2.7 Virus titration and plaque purification: Serial dilutions of recombinant virus were used to infect 9 x 10^5 Sf9 cell monolayers in 35mm wells. After 1 to 2 h at room temperature, the supernatant was replaced with molten 3% low melting agarose diluted 50% with Grace's medium at 37°C. Following incubation at 28°C for 4 days, 1ml 1mg/ml Neutral Red diluted 10X with molten 1.5% low melting agarose was added to the wells and incubated for a further day. Individual plaques were counted and picked with a 1ml micropipette tip and added to 1ml Grace's medium.

2.2.8 Protein labelling: Monolayers of Sf9 cells in 16mm wells were infected with wild type or recombinant baculoviruses at an MOI of 10 pfu/cell. After 1 h, the inoculum was replaced with Grace's medium and incubated at 28°C for 30-32 h. The medium was replaced with 500µl methionine-free Eagles' medium and incubated at 28°C for 1 h. The starving medium was replaced with 200µl fresh methionine-free Eagles' medium to which approximately 10µCi ^{35}S-methionine per well had been added. The cells were harvested after a further 16 h incubation at 28°C and resuspended in 60µl 1X PSB.

2.2.9 VP1 solubility assays: VP1 recombinant baculovirus-infected cells were harvested, washed in 1X PBS and concentrated by low speed centrifugation. Cells were suspended at 1 x 10^8 cells/ml in 0.15M STE-TX buffer (0.01M NaCl, 10mM Tris-HCl pH7.4, 1mM EDTA, 0.5% or 1% Triton X-100) and incubation at room temperature for 10 min. Nuclei were removed together with the insoluble fraction by centrifugation at 1800g for 5 min and washed once with half the original volume of 0.15M STE-TX. The supernatants were combined and diluted 50% with 2X PSB, and the pellets were resuspended in an equal final volume 1X PSB.

2.3 Bacterial expression:

2.3.1 Cells: *E. coli* BL21(DE3), obtained from Prof. LA Ball, and DH5α cells were used. BL21(DE3) cells allow high efficiency protein expression of genes cloned into expression vectors under the control of the bacteriophage T7 promoter. The T7 RNA polymerase gene is carried on the bacteriophage λ DE3, which is integrated into the chromosome of BL21.

2.3.2 Vectors: Plasmids 91(8,4)p, 91(2,0)p and 91ΔK(2,0)p have been previously described. Plasmids pPROEX HTa, b and c (Gibco BRL) were kindly provided by Prof. H Huismans of the Department of Genetics at the University of Pretoria.

2.3.3 Plasmid constructions: The AHSV-9 VP1 gene open reading frame was subcloned from 91orf.pFB-HT into pPROEX Hta with *Nco* I and *Sac* I to yield 91orf.pPROEX-HT.

2.3.4 Expression: Plasmids 91(8,4)p, 91(2,0)p and 91ΔK(2,0)p were transformed into BL21(DE3) cells by standard methods. Cultures were grown to an OD_{550} of 0.3 – 0.5 prior to induction of expression by addition of IPTG to 1mM.

Expression from 91orf.pPROEX-HT was similarly achieved, according to the manufacturer's instructions. This involved transformation into DH5α cells, incubation to an OD_{590} of 0.5 – 1.0 prior to addition of IPTG to a final concentration of 0.6mM. Non-recombinant vectors served as negative controls.

2.3.5 Expression analysis: Samples taken pre- and 1, 2 or 3 h post-induction were precipitated, lysed and analysed by standard SDS-PAGE.

3. RESULTS

3.1 vTF7-3 driven *in vivo* gene transcription and expression

The VACV bacteriophage T7 DNA-dependent RNA polymerase recombinant system for expression of foreign genes *in vivo* has previously been used to demonstrate sequence-specific replication by a viral RNA polymerase, specifically that of FHV (Ball 1994). In order to investigate the application of this system to AHSV replication, it was hypothesised that, in addition to VP1

(encoded by AHSV genome segment 1), VP3 (encoded by AHSV genome segment 3) may also be required for replicase activity of AHSV-specific RNA. This hypothesis was based on results obtained with rotavirus replication (Patton *et al.* 1997), which indicated a prerequisite for co-representation of the polymerase and the core shell protein. A clone of AHSV genome segment 8 was also procured to serve as a possible alternative (shorter) template for replication, and for investigation of a possible role of the RNA-binding NS2 in replication.

3.1.1 **Vector constructs**

An exact cDNA copy of the AHSV-9 genome segment 1, without extraneous sequences, was PCR amplified from the cloned full-length gene (91.pBR322) using terminal sequence-specific primers. A precise blunt-ended amplicon of the AHSV-9 genome segment 8 (encoding NS2) was similarly prepared. A blunt-ended cDNA copy of the open reading frame of the AHSV-9 VP3 gene with non-AHSV-specific termini was isolated by enzymatic excision and terminal fill-in. These genes were cloned into V(2,0), a vector for insertion of foreign genes under the control of the bacteriophage T7 DNA-dependent RNA polymerase promoter, with a downstream HDV ribozyme sequence for self-cleavage of transcribed RNA. The constructs were designed to serve as a source of both mRNA for expression of VP1, VP3 and NS2 and competent genome segment 1 and 8 RNA templates for AHSV-specific replication. The vector-labelling convention uses the numerals within parentheses to indicate extraneous nucleotides remaining at the 5' and 3' termini respectively of T7 RNA polymerase-initiated transcripts after ribozyme-mediated cleavage. Thus, V(2,0) contained 2 nucleotides (GG) between the major site of transcriptional initiation and the *Stu* I site for foreign gene insertion, and the *Sma* I site for foreign gene insertion coincidental with the site for ribozyme-mediated self-cleavage. Clones were labelled as ab(x,y)p or ab(x-x_1,y-y_1)n, where a refers to the AHSV serotype and b to the relevant genome segment, x indicates the number of nucleotides between the site of transcriptional initiation and the first nucleotide of the inserted gene sequence and y the number of nucleotides between the end of the inserted gene sequence and the site of autolytic cleavage. x_1 and y_1 indicate, where relevant, the number of 5' or 3' terminal AHSV-specific non-coding nucleotides deleted from processed transcripts respectively. p (positive) or n (negative) indicates the sense of the transcript, pertaining to the AHSV-specific

protein coding. Negative-sense clones were prepared either as negative controls (absence of expressed protein) or to serve as source for negative-sense templates for replication. The junction sites of every clone were analysed by DNA sequencing. The clones obtained are described in Table 4.1.

The inserts in all AHSV-9 segment 1 clones, except one, were derived by PCR amplification with high fidelity *Pwo* polymerase. In the case of 91(8,4)p, the possibility of *Taq* polymerase-induced errors in the VP1 gene sequence advocated the replacement of the majority of the coding region of the PCR-tailored gene of 91(8,4)p with cDNA from a9s1.pBR. The integrity of the remaining DNA was confirmed by sequencing. This plasmid served as a control source for the expression of authentic AHSV VP1. In addition, this clone was designed to overcome the possible detrimental effect of uridylate residues downstream of the initiation site for T7 RNA polymerase, as documented by Ling *et al* (1989), by the inclusion of a 5' uridylate-poor extension.

An additional control in the form of 91ΔK(2,0)p was constructed to serve as source of shorter (2075 bp) replication-competent transcripts encoding deletion mutant VP1. The open reading frame of AHSV-9 VP1 was modified to eliminate codons 218 through 847, such that the mutated gene encoded a protein of 675 amino acids (called VP1mut), with a predicted molecular weight of 78.5kDa.

3.1.2 *In vitro* transcription and translation

T7 RNA polymerase-driven transcription efficiency and ribozyme-mediated self-cleavage activity was analysed by *in vitro* transcription of approximately equal quantities of *Bam* HI-linearised and circular or *Nco* I-mock digested 91(8,4)p and 91(2,0)p (Figure 4.2). Linear DNA appeared to be transcribed more effectively than circular DNA, although a considerable amount of nucleic acid, that was presumably protein-bound, from the transcription of circular templates was held back in the wells of the agarose gel. It would appear that inefficient transcriptional termination and incomplete ribozyme-mediated self-cleavage, as well as low levels of non-linearised plasmid, led to some background run-through transcripts. No significant difference in the yields of *in vitro*-generated transcripts from 91(8,4)p or 91(2,0)p could be detected.

In vitro translation of positive-sense transcripts with wheat germ extract demonstrated the expression of full-length (150kDa) VP1 (Figure 4.3). The

Table 4.1 AHSV genes cloned into V(2,0). The labeling of the clones is described in the text. The 5' and 3' terminal sequences of ssRNA derived from T7 RNA polymerase-driven transcription are based on sequence data of the junction sites of vector and insert. AHSV-specific sequences are highlighted in bold, hyphens delineating terminal extensions of vector or other origin.

Gene	Clone	RNA terminal sequences	Comments
AHSV-9 VP1	91(8,4)p	GGGGGATT-**GTTTAT**.. ..**ACTTAC**-AATC	cDNA clone; preferred transcription initiation sequence
	91(2,0)p	GG-**GTTTAT**.. ..**ACTTAC**-	exact 3' terminus
	91ΔK(2,0)p	GG-**GTTTAT**.. ..**ACTTAC**-	*Kpn* I_{667} - *Kpn* I_{2557} deleted; exact 3' terminus
	91(2,0-3)p	GG-**GTTTAT**.. ..**ACT**-	3' terminus deleted
	91(2,0)n	GG-**GTAAGT**.. ..**ATAAAC**-	(-) sense RNA; exact 3' terminus
AHSV-9 VP3	93(7-26,5-43)p	GGGATCT-**ATG**CAA.. ..**TAG**CAGCC-AGATC	start and stop codons underlined; complete open reading frame
	93(7-43,5-26)n	GGGATCT-**GGCTGC**.. ..**TTGCAT**-AGATC	(-) sense RNA
AHSV-9 NS2	98(2,0)p	GG-**GTTTAA**.. ..**ACATAC**-	exact 3' terminus
	98(2,0)n	GG-**GTATGT**.. ..**TTAAAC**-	(-) sense RNA; exact 3' terminus

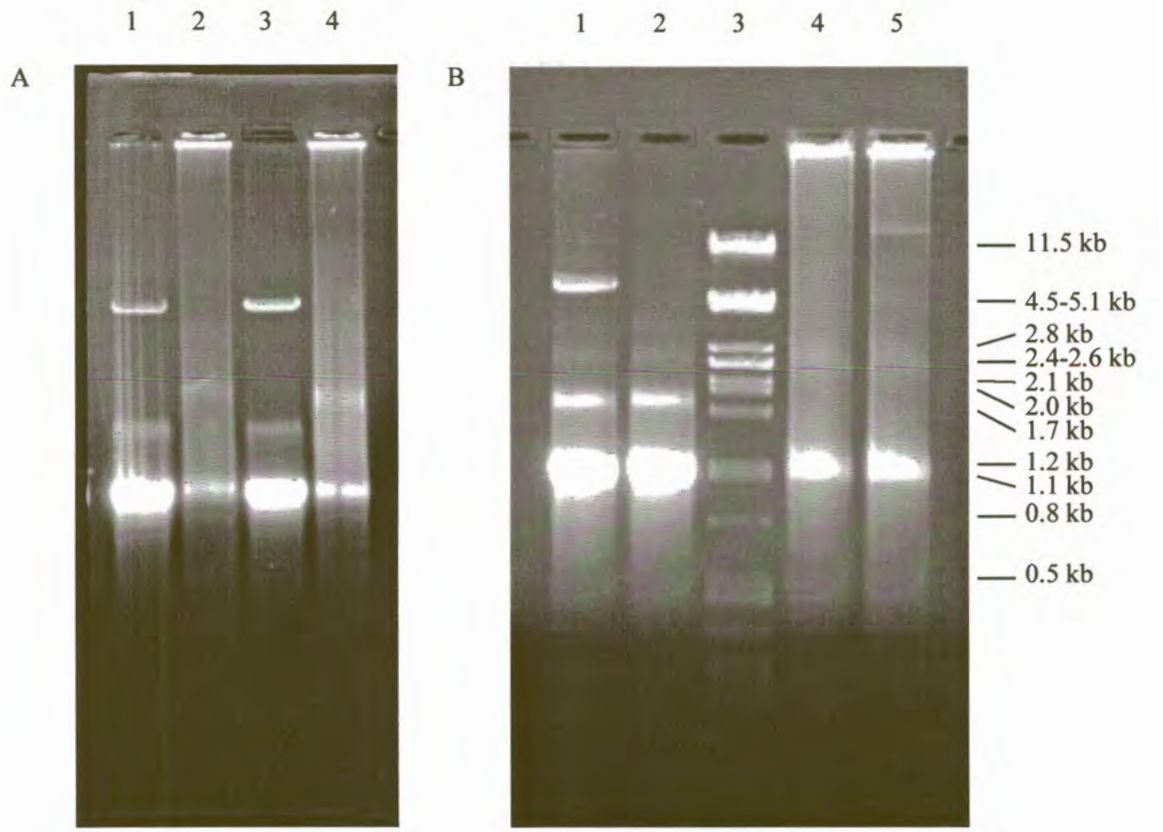

Figure 4.2 Agarose gel electrophoretic analyses of *in vitro* transcriptions of recombinant AHSV-9 VP1 constructs cloned into V(2,0) ribozyme vector plasmid.

A) 91(8,4)p (lanes 1 and 2) and 91(2,0)p (lanes 3 and 4) were linearised with *Bam* HI prior to transcription (lanes 1 and 3) or left undigested (lanes 2 and 4).

B) 91(8,4)p was digested with *Bam* HI (linearised; lanes 1 and 2) or *Nco* I (non-linearised; lanes 4 and 5) prior to transcription. Transcription products were analysed directly (lanes 1 and 5) or treated with Dnase I prior to analysis (lanes 2 and 4). Lane 3 represents $\lambda_{Hind\ III}$ dsDNA size marker (labelled on the right).

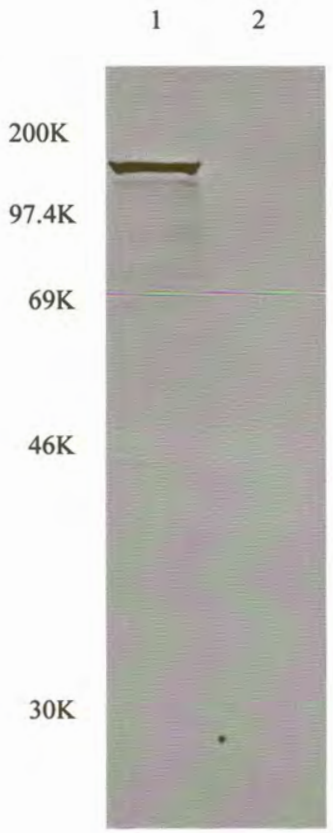

Figure 4.3 An autoradiograph of an SDS-PAGE analysis of *in vitro* translated AHSV-9 VP1 (lane 1). *In vitro* translation of the same gene in the reverse orientation was used as a negative control (lane 2). Molecular weight markers are indicated on the left.

observed doublet is thought to be an artefact of the *in vitro* translation system used. The slightly lighter larger band represents full-length VP1 when compared to subsequently *in vivo* expressed protein (results not shown). Once again, as found with AHSV-1 VP1 (chapter 2), no product could be obtained with the rabbit reticulocyte lysate *in vitro* translation system.

3.1.3 *In vivo* expression

The integrity of the open reading frames of the genes and functionality of the ribozyme in all positive-sense clones was confirmed by *in vivo* labelling of expressed proteins in vTF7-3 infected cells transfected with the relevant plasmid. Herein, T7 RNA polymerase-driven transcripts function as mRNA for translation of the encoded protein. Expression of AHSV-9 VP1, VP1mut and VP3 was shown (Figure 4.4). The level of VP1 expressed from 91(8,4)p was considerably greater than that expressed from the 91(2,0)p template. The gene product of 91ΔK(2,0)p (VP1mut) appeared on SDS-PAGE as a protein of approximately 100kDa in contrast to the predicted molecular weight of 78.5kDa. In the case of AHSV NS2, labelled protein could not be distinguished from background proteins by SDS-PAGE. Accordingly, circular 98(2,0)p plasmid was transcribed *in vitro* with T7 RNA polymerase, and the resultant RNA translated with the rabbit reticulocyte *in vitro* translation system. SDS-PAGE analysis and autoradiography (Figure 4.5) confirmed the synthesis of full-length protein.

3.1.4 *In vivo* polymerase assay

Hereafter, various combinations of the cloned genes (as shown in Table 4.2) were transfected singly or jointly into vTF7-3 infected cells, and the cells were screened for AHSV-specific RNA replication as prescribed by Ball (1992) and described in Materials and Methods. In these assays, ribozyme-mediated cleavage of the primary T7 RNA polymerase-driven transcripts yields RNA molecules with precise AHSV-specific 3′ termini to function as potential templates for AHSV-specific replicase activity. The addition of actinomycin D served to inhibit DNA-dependent RNA polymerase activity prior to radioactive labelling. Hence, if the transfected cells expressed active AHSV replicase and if the cleaved transcripts were competent templates for replication, cRNA would be synthesised in an RNA-dependent, actinomycin D-resistant reaction. If, in turn, the cRNA was a competent template, full RNA replication would ensue.

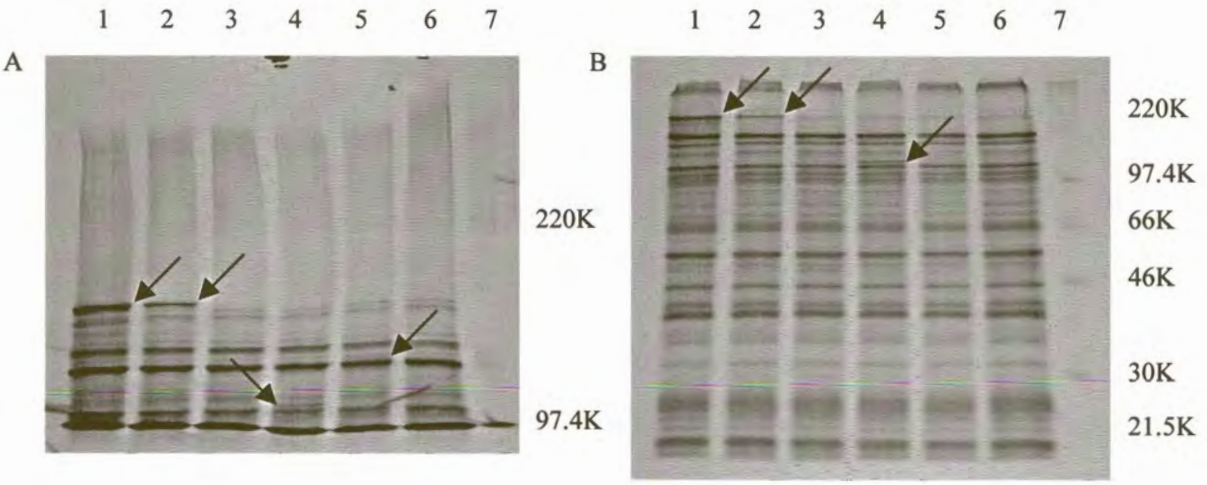

Figure 4.4 Autoradiographs of SDS-PAGE analyses, by longer (A) or shorter (B) electrophoretic separation, of *in vivo* translated recombinant AHSV-9 proteins. The lanes represent samples of vTF7-3 infected cells transfected with 91(8,4)p, 91(2,0)p, V(2,0), 91ΔK(2,0)p, 93(7-26,5-43)p and 98(2,0)p (lanes 1 to 6 respectively), expressing VP1 (lanes 1 and 2), a VP1 deletion mutant (lane 4), VP3 (lane 5) and NS2 (lane 6). Lane 3 represents a negative control. The expressed AHSV-specific proteins are indicated with arrows. NS2 (lane 6) is not visible. The molecular weight markers (lane 7) are labelled on the right of each autoradiograph.

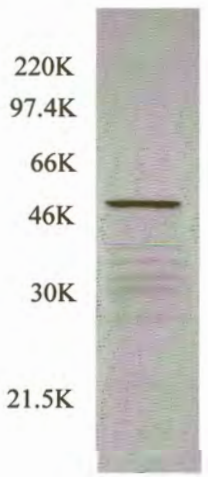

Figure 4.5 Autoradiograph of SDS-PAGE analysis of *in vitro* translated AHSV-9 NS2. The positions of the molecular weight markers are indicated on the left.

Table 4.2 Summary and combinations of AHSV gene clones transfected into vTF7-3 infected BSC 40 cells.

	91(8,4)p	91(2,0)p	91(2,0-3)p	91ΔK(2,0)p	91(2,0)n	93(7-26,5-43)p	93(7-43,5-26)n	98(2,0)p
Gene product	VP1	VP1	VP1	VP1mut	-	VP3	-	NS2
Replication-competent transcript	X?	✓	?	✓	✓	X	X	✓
Combination								
1	✓							
2		✓						
3			✓					
4					✓			
5						✓		
6							✓	
7	✓	✓						
8	✓					✓		
9		✓				✓		
10		✓						✓
11		✓				✓		✓
12				✓		✓		✓

Thus, the assay sought to identify AHSV VP1-dependent RNA-dependent RNA polymerase activity through actinomycin D-resistant synthesis of radioactively labelled RNA. A standard incubation temperature of 28°C was used throughout for analysis of polymerase activity based on reports in the literature regarding the optimal temperature for activity of the BTV RNA polymerase in an *in vitro* assay with core AHSV particles (Van Dijk & Huismans 1982). However, upon formaldehyde agarose gel electrophoresis and fluorography of isolated RNA, no evidence for AHSV VP1-dependent RNA-dependent RNA polymerase activity could be found, based on the lack of detectable ^{3}H-labelled RNA resulting from the replication of a competent RNA template by active VP1 in the presence of actinomycin D. Smears visible on the fluorographs in certain cases (Figure 4.6) appeared not to be correlated to the presence or absence of a particular AHSV protein, being particularly evident in transfections with 91(8,4)p and to a lesser extent with 93(7-43,5-26)n. These smears were shown not to be the result of AHSV RNA-specific replication, as only transcript-specific RNA could be detected by hybridisation of the isolated RNA to strand-specific ^{32}P-labelled AHSV RNA probes (Figure 4.7). Initial promising hybridisation results which suggested possible low levels of AHSV-specific replication in the presence of 91(2,0)p could not be confirmed by repetition. In fact, repetitions with control plasmids, such as 91ΔK(2,0)p, revealed occasional but obstinate low level cross contamination of samples (results not shown).

The application of this system in our laboratory was controlled by reproduction of the self-directed replication of FHV RNA 1, and replication of FHV RNA 2, as described by Ball (1994). RNA replication of FHV genomic RNA 1 and RNA 2 is catalysed by highly active RNA-dependent RNA polymerase encoded by RNA 1. During RNA 1 replication, a small sub-genomic RNA (RNA 3) is also synthesised. In this case, the RNA replicase encoded by transcripts of transfected FHV1(1,0) was demonstrated to be capable of authentically replicating plasmid-derived transcripts of FHV RNA 1 and 2 in the presence of actinomycin D. This was achieved by RNA analysis following transfection of combinations of FHV1(1,0) and FHV2(0,0) in vTF7-3 infected cells, which yielded distinct bands representing FHV RNA 1, 2 and 3 replicons (Figure 4.8). In line with the findings of Ball (1994), synthesis of RNA 3 was suppressed by the replication of RNA 2.

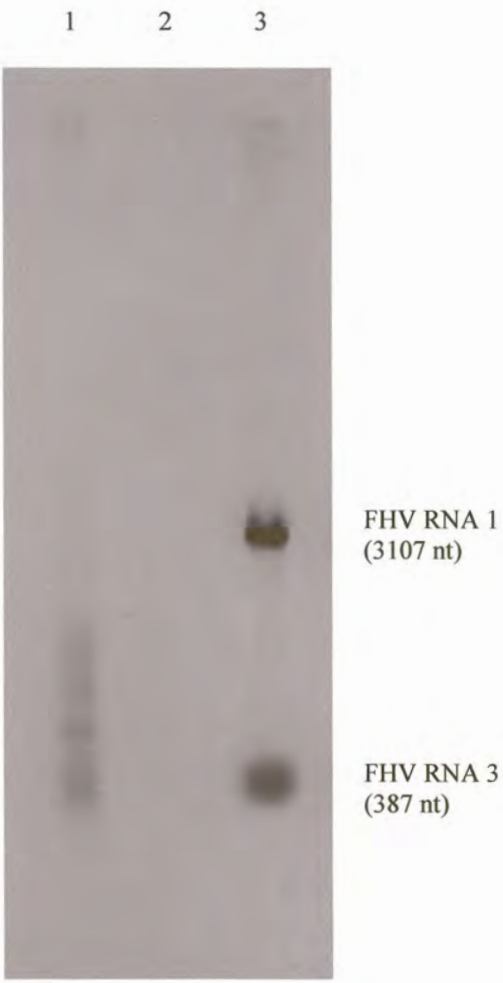

Figure 4.6 Fluorograph of agarose-formaldehyde gel electrophoretic analysis of labelled cytoplasmic RNAs in vTF7-3 infected cells, transfected with 91(8,4)p (lane 1) or FHV1(1,0) (lane 3). Lane 2 represents an untransfected control. Labelling with ^{3}H-uridine occurred at 21 hours post transfection in the presence of actinomycin D. The FHV RNA species (labelled on the right) serve as positive control and size marker.

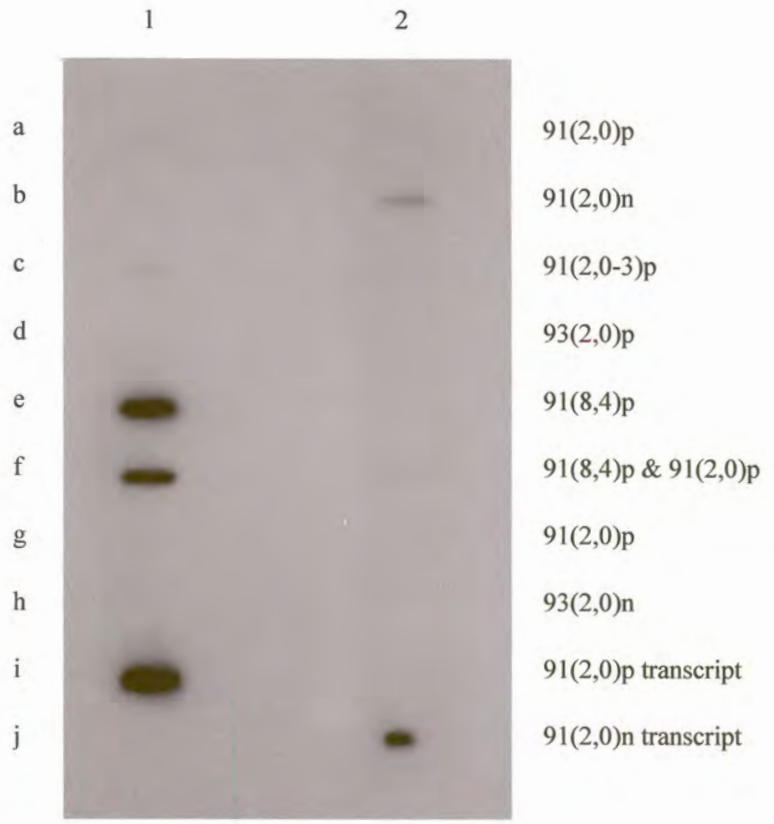

Figure 4.7 Autoradiograph of northern slot blots probed with ^{32}P-ATP labelled AHSV-9 segment 1 positive-sense (column 1) or negative-sense (column 2) *in vitro* transcripts. Rows a to h represent cytoplasmic RNAs isolated from vTF7-3 infected cells transfected with the plasmids indicated on the right, whereas positive- and negative-sense *in vitro* RNA transcripts were blotted in rows i and j respectively as controls. Bands are visible on the original autoradiograph in rows a, c, e, f and i in column 1 and rows b, f and j in column 2.

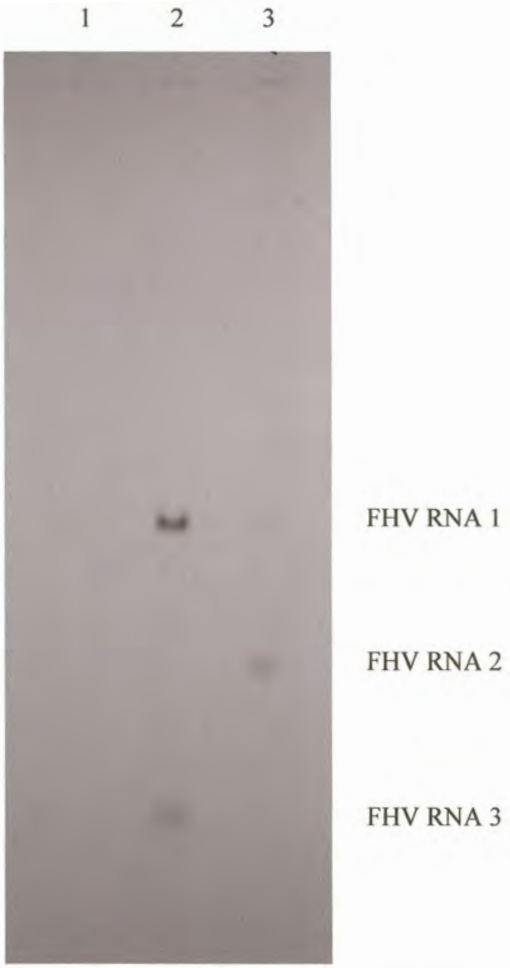

Figure 4.8 Fluorograph of agarose-formaldehyde gel electrophoretic analysis of labelled cytoplasmic RNAs in vTF7-3 infected cells, untransfected (lane 1) or transfected with FHV1(1,0) (lane 2) or FHV1(1,0) and FHV2(0,0) (lane 3). Labelling with ^{3}H-uridine occurred at 21 hours post transfection in the presence of actinomycin D. The FHV RNA species are labelled on the right.

3.2 Baculovirus expression of AHSV VP1

The Bac-to-Bac baculovirus expression system is based on site-specific transposition of an expression cassette from recombinant donor plasmid into a baculovirus shuttle vector (bacmid) propagated in *E. coli* DH10Bac as a large plasmid.

3.2.1 Construction of recombinant baculoviruses

Recombinant bacmids containing the AHSV-9 VP1 gene were constructed according to the manufacturers' instructions and as described in Materials and Methods to express VP1 as wild type protein or as a fusion protein with an amino-terminal histidine tag. The latter required PCR modification of the AHSV genome segment 1 VP1 gene to remove a stop codon in the 5' non-coding region. A novel *Bgl* II restriction enzyme site, presumably introduced during PCR amplification, was detected near to the 3' terminus of the cloned gene. Insertion of the VP1 gene into the bacmid DNA was verified by PCR analysis (Figure 4.9). The recombinant bacmids were transfected into insect cells. Viral stocks harvested from the transfected cells were used to infect cells for subsequent protein expression.

3.2.2 VP1 expression

Initially, expression was confirmed by *in vivo* radiolabelling, demonstrating expression of a 150kDa protein from both wild type and fusion protein constructs (Figure 4.10). The expression yield from the former was clearly superior, prompting titration of this recombinant baculovirus. This action was also supported by possible mutations in the PCR-derived fusion protein gene. Individual plaques were picked and screened for VP1 expression by *in vivo* radiolabelling and/or Coomassie staining of SDS-PAGE analyses of infected cell lysates. 9 out of 10 plaques screened displayed high levels of VP1 expression. 5 of these were passaged 4 times to investigate stability of expression levels. VP1 was expressed at maximum levels in the second and third passages (approximately 5-10μg/10^6 cells) but the level of expression was slightly reduced in the fourth passage.

It should be noted that re-infection of cells with recombinant AHSV VP1 baculoviral stocks stored at 4°C or -70°C for a number of weeks was found to

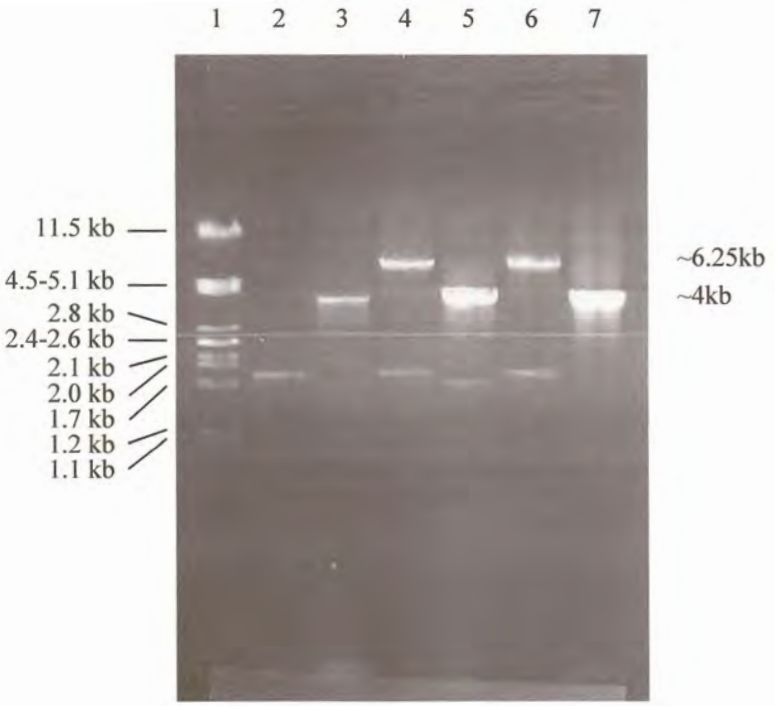

Figure 4.9 PCR analysis of bacmid transposed with AHSV-9 genome segment 1 cDNA recombinant pFastBac1 (lanes 2 to 5) or pFastBacHT (lanes 6 and 7). Putative recombinants were amplified with the pUC/M13 forward and reverse primers (lanes 2, 4 and 6) and AHSV-9 genome segment 1 termini-specific primers (lanes 3, 5 and 7). The former primers are directed at sequences on either side of the transposon attachment site on the bacmid, yielding amplicons of 2300bp or 2430bp (excluding gene insertions) from bacmids transposed with pFastBac1 or pFastBacHT respectively. Lane 1 represents $\lambda_{Hind\ III}$ size marker (labelled on the left). The sizes of the relevant PCR amplicons are indicated on the right. Lane 2 was negative for recombinant bacmid DNA. Low level non-specific background amplification is visible.

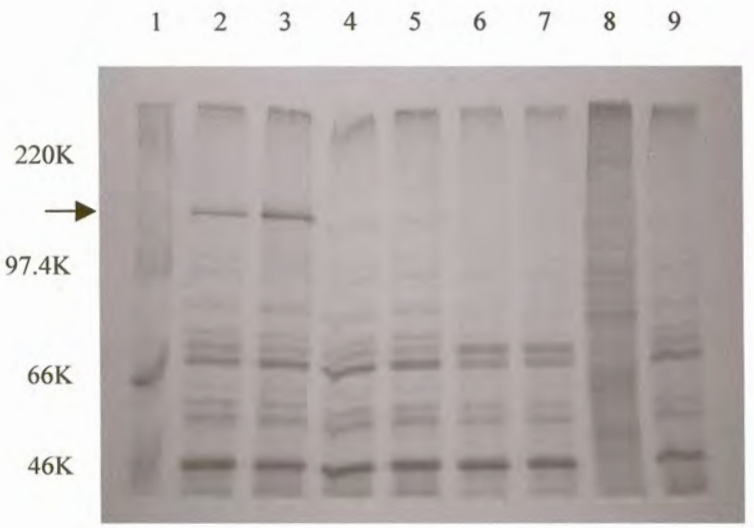

Figure 4.10 Autoradiograph of SDS-PAGE separated extracts of putative wild type (lanes 2 and 3) or histidine-tagged (lanes 4 to 7) AHSV-9 VP1 recombinant baculovirus-infected cells. Lanes 8 and 9 represent mock- and wild type baculovirus-infected cells respectively. The molecular weight markers are labelled on the left and the position of expressed VP1 is indicated with an arrow.

yield little or no detectable VP1 by Coomassie staining. Additional passaging of these stocks appeared to result in total loss of detectable VP1 expression, even though evidence of viral infection through the presence of viral proteins in infected cell lysates and the reduction of cellular proteins could be clearly observed. In addition, further attempts to repeat generation of recombinant AHSV VP1-expressing baculoviruses proved unsuccessful, with no recombinant protein detectable on Coomassie-stained SDS-PAGE gels of lysates of cells infected with viruses harvested from cells transfected with recombinant bacmids.

3.2.3 **VP1 solubility assays**

Solubility assays of recombinant baculovirus-expressed VP1 were performed by treatment of infected cells with varying concentrations of Triton X-100. 100% of the expressed VP1 was found to pellet during 1800g centrifugation (Figure 4.11).

3.3 Bacterial expression of AHSV VP1

Attempts at bacterial expression of full-length AHSV-9 VP1 proved unsuccessful. Neither 91orf.pPROEX-HT nor inducible T7 RNA polymerase-driven expression from 91(8,4)p and 91(2,0)p in BL21(DE3) cells yielded detectable recombinant protein compared to wild type negative controls on Coomassie-stained SDS PAGE gels.

4. DISCUSSION

4.1 vTF7-3 driven *in vivo* gene transcription and expression

The *in vivo* replication system using vTF7-3 for expression of foreign genes on transfected plasmids as described represents extremely powerful technology. The *in vivo* nature of the assay at least partially simulates the naturally virus-infected cell, providing possible essential cellular cofactors for replication, and additional or mutated genes can simply be subcloned into a bacterial plasmid vector for incorporation into the assay and analysis. The

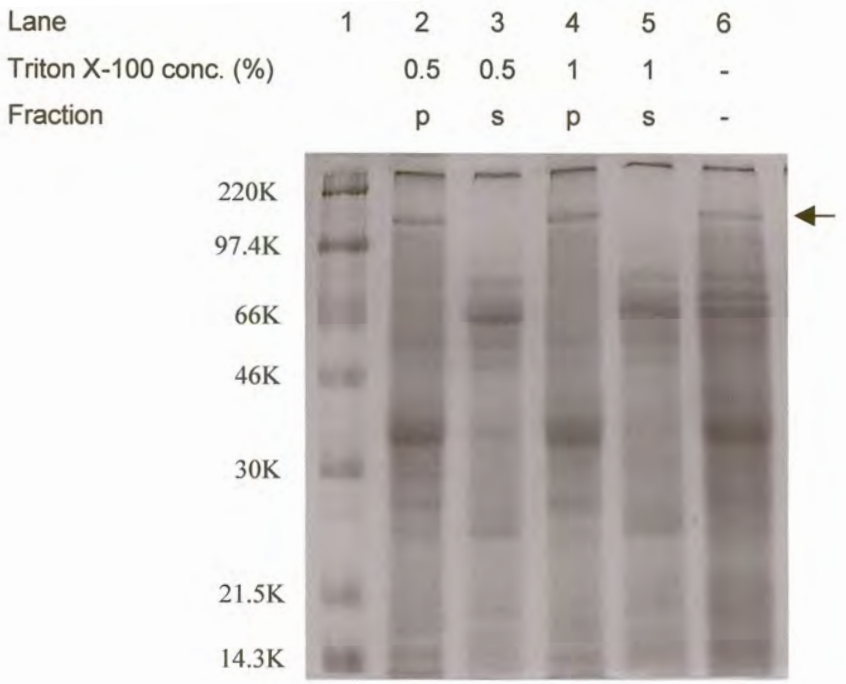

Figure 4.11 Solubility assays of recombinant baculovirus-expressed AHSV-9 VP1. Recombinant baculovirus-infected cells were harvested and treated with 0.5% or 1% Triton X-100 prior to low speed centrifugation. Pellets (p) and supernatants (s) were analysed by SDS-PAGE and Coomassie staining. Lane 6 represents unfractionated cell extracts. The size markers (lane 1) are labelled on the left. The position of AHSV VP1 is indicated with an arrow.

investigation described here aimed to investigate the capacity of this approach for research into AHSV replication. However, due to the relative complexity of AHSV compared to the FHV model, possible alternative approaches involving high level VP1 expression were also envisaged.

In vivo vTF7-3 assisted assays for RNA polymerase activity were carried out with AHSV-9 VP1. Based on reports that both the rotavirus RNA polymerase and the core shell protein are essential for replicase activity (Patton *et al.* 1997), AHSV-9 VP3 was also included in the assay. Reports of the open reading frame of rotavirus mRNAs specifically promoting the synthesis of dsRNA and a reported inverse correlation between replication efficiency and the length of the RNA template (Patton *et al.* 1999) were also taken into account. Hence, full-length cDNA of genome segments 1 and 8 of AHSV-9, encoding VP1 and NS2 respectively, were obtained and cloned into a vector suitable for generating RNA with authentic 3' termini following *in vivo* transcription in vTF7-3 infected cells. T7 RNA polymerase-driven transcription of the foreign genes in this vector effected the integration of two additional 5' G residues on the mRNA, thus detracting from the authenticity of the 5' termini. It was envisaged that these RNAs would serve as templates for AHSV VP1-dependent replication. In addition, the coding region of AHSV-9 genome segment 3, lacking the non-coding termini, was cloned into this vector as a source of non-authentic mRNA encoding VP3. Transcription and translation of the genes *in vivo* was verified by radioactive labelling and SDS-PAGE analysis, or *in vitro* in the case of NS2.

Transcription and expression of the AHSV-9 VP1 gene alone or in conjunction with the VP3 gene yielded no detectable RNA-dependent RNA polymerase-dependent replication of AHSV-9 segment 1 mRNA on agarose-formaldehyde gels. Inclusion of the AHSV-9 NS2 gene in the assay as an alternative shorter template for replication similarly yielded negative results. It was suspected that smears that were obtained on fluorographs following formaldehyde agarose gel electrophoresis with certain samples related to T7 RNA polymerase-dependent transcriptional breakthrough. Laboratory application of the assay was confirmed using the FHV template-dependent replication (Ball 1994) as positive control.

In an attempt to improve the sensitivity of the AHSV assay, isolated RNA was subjected to Northern hybridisation with corresponding specific probes. No

evidence of replication through the presence of negative-strand RNA could be found.

Ribozyme cleavage of RNA transcripts yields a 2'3'-cyclic phosphate 3' terminus as opposed to the natural 3'-OH terminus (Long & Uhlenbeck 1993). Although the ribozyme-cleaved cDNA transcripts of FHV were shown to replicate with comparable efficiency to wild type RNA (Ball & Li 1993), it is not known what effect this may have on AHSV VP1-specific replication.

It should also be noted that the two additional G residues at the 5' end of transcribed RNA might play a role in preventing, or reducing, template-specific replication. In the case of FHV, the 5' termini of the genome segments RNA 1 and RNA 2 are GUUUUGA and GUAAAC respectively. Authentic RNA transcripts with no additional 5' nucleotides were found to replicate to approximately ten times higher levels than transcripts with 5' extensions of one or two G residues (Ball & Li 1993). On the other hand, plasmids with one or two additional 5' G residues at the T7 polymerase initiation site were transcribed much more efficiently (Ball & Li 1993; Ball 1995).

Ling *et al.* (1989) have previously documented the negative influence of uridylate residues downstream of the T7 RNA polymerase initiation site on transcription, as also confirmed by Ball and Li (1993). The extensive representation of such residues in all AHSV genome segment 5' sequences ($GUU^{A}_{U}A^{A}_{U}$) prompted the selection of a vector possessing two additional G residues at the T7 polymerase initiation site. *In vivo* transcription of all AHSV-specific constructs prepared during this study was established by verifying expression of the encoded proteins. It was also demonstrated that higher expression levels of VP1 were obtained from vectors in which fewer uridylate residues were present downstream of the T7 RNA polymerase initiation site.

Any assay investigating viral replication demands the consideration of a wide array of determinants that may play a role. Some of the most obvious elements include the components of an active replication complex, the level of replicase expression and activity and possible inhibitory factors such as the high concentrations of actinomycin D, temperature-dependence etc.

In contrast to the vast majority of cellular proteins, proteins of the *Reoviridae* are translated from capped but non-polyadenylated mRNAs. Vende *et al.* (2000) recently demonstrated that NSP3 plays a role in the enhancement of rotavirus mRNA translation through interaction with the eucaryotic translation

initiation factor eIF4G and the 3' end of the mRNA, again highlighting the importance of the non-structural proteins in virus replication. Similar inferences with AHSV may have a bearing on and would be interesting to investigate in the assay under discussion.

Previous work on the effect of temperature on the *in vitro* transcriptase reaction of orbiviruses (Van Dijk & Huismans 1982) indicated a low temperature optimum of 28°C. However, it was pointed out that the low temperature preference of the viral transcriptase was not necessarily an inherent characteristic of the enzyme itself. Indeed, virus propagation in cell culture is carried out at 37°C.

Unfortunately, institutional reorganization prevented further investigation of the *in vivo* assay for AHSV VP1 activity.

4.2 Baculovirus expression

Baculovirus expression vectors have been widely employed to produce high levels of accurately processed and biologically active proteins. In the case of the *Reoviridae*, multiple genes have been expressed by recombinant baculoviruses and found to be biologically active, including the RNA polymerases of BTV (Urakawa *et al.* 1989) and rotavirus (Chen *et al.* 1994b).

The expression of foreign genes in baculovirus vectors is usually achieved under polyhedrin promoter control (O'Reilly *et al.* 1992). The polyhedrin protein is produced at very high levels in the nuclei of baculovirus-infected cells in the late phase of infection and accumulates to form inclusion bodies that contain embedded virus particles. As such, the protein is dispensable for virus replication and only plays a role in the occlusion phase of baculovirus infection. The BAC-TO-BAC (Gibco BRL) baculovirus expression systems are suitable for generating recombinant baculoviruses expressing foreign genes under control of the polyhedrin promoter by site-specific transposition in *E. coli* (Luckow *et al.* 1993). The pFastBac plasmids contain all the elements necessary for cloning and subsequent transfer to the baculovirus genome (bacmid DNA). The transfer of the expression cassette in the recombinant pFastBac plasmid to the bacmid DNA occurs in DH10Bac cells by Tn7 site-specific transposition.

Utilising this sytem, VP1 of AHSV-9 was shown to be expressed in recombinant baculovirus-infected cells as a 150kDa protein by radiolabelling and

on Coomassie blue-stained SDS-PAGE gels. Both wild type VP1 protein and VP1 protein with an amino-terminal histidine tag were expressed. The lack of availability of anti-AHSV serum containing sufficient titres of anti-VP1 antibodies for detection prevented the use of Western immunoblotting or immunoprecipitation studies to confirm the identity of the expressed protein. Similarly, anti-BTV serum was found not to react with either BTV virion VP1 nor baculovirus-expressed BTV VP1 (Urakawa *et al.* 1989; Loudon & Roy 1991). In these cases, the dilemma was resolved by preparing rabbit monospecific serum against purified baculovirus-expressed BTV VP1, which was then shown to bind to BTV virion VP1.

Four rounds of passaging with maintenance of high expression levels of the wild type AHSV VP1 suggested a stable recombinant baculovirus. However, storage of the virus appeared to abolish VP1 expression upon reinfection. No satisfactory explanation of this outcome or the apparent disparity has been found. Lack of funding support and infrastructure necessitated termination of further attempts.

Expression of high levels of AHSV VP1 opens possibilities for the investigation of RNA binding capacity, interactions with other viral proteins and polymerase activity.

However, high level expression of proteins may eventuate insolubility and hence inactivity. Solubility assays of recombinant baculovirus-expressed AHSV-9 VP1 through differential centrifugation of cell lysates suggested absolute insolubility of the protein. Similarly, baculovirus-expressed VP1 of rotavirus was recovered from the pellet following low speed centrifugation of infected cells lysed with 0.1% deoxycholate (Patton *et al.* 1997). Nonetheless, recovered rotavirus VP1 replicated RNA when supplemented with purified core protein VP2. On the other hand, 30% of baculovirus-expressed BTV VP1 was reportedly solubilized and present in the cell lysate supernatant used for polymerase activity assays (Urakawa *et al.* 1989). In the case of *Bovine viral diarrhea virus* (BVDV), full-length NS5B protein expressed in insect cells by recombinant baculovirus was shown to possess RNA-dependent RNA polymerase activity when solubilized by high concentrations of detergent, salt and glycerol (Zhong *et al.* 1998). It was subsequently also demonstrated that removal of a C-terminal hydrophobic domain from BVDV NS5B yielded soluble protein with RNA-dependent RNA polymerase activity when expressed in *E. coli* (Lai *et al.* 1999).

In a recent report, 45kDa dsRNA bacteriophage ϕ6 polymerase protein expressed to high levels in bacteria was found to be in an insoluble form (Makeyev & Bamford 2000). However, expression at low temperatures yielded detectable levels of soluble and active protein, leading to the first description of template-dependent RNA synthesis by an isolated polymerase protein of a dsRNA virus.

4.3 Bacterial expression

Bacterial expression of AHSV VP1 has thus far been unsuccessful. Similar difficulties with the expression of large viral proteins in bacteria have been described by other researchers, such as with the expression of *Bamboo mosaic virus* (BaMV) RNA-dependent RNA polymerase (Li *et al.* 1998). Open reading frame 1 (encoding a 155kDa viral protein containing methyltransferase, RNA helicase and polymerase motifs) of BaMV, a positive-sense RNA virus in the potexvirus group, was inserted into four different bacterial and one baculoviral expression vector (Li *et al.* 1998). The expression was found throughout to be "low or even barely detectable", except when expressed with thioredoxin fused at its amino terminus, using pET-32a vector, in *E. coli* BL21(DE3) cells. This purified fusion protein was shown to be able to generate a ^{32}P-labelled RNA product complementary to 3'-terminal viral RNA fragments of the positive- or negative-strand in *in vitro* polymerase assays. Several hypothetical reasons were proposed for the limited heterologous expression. The efficiency of the read-through translation of the 155kDa viral protein may have been poor and the incompletely translated polypeptides degraded by proteolytic action. It was also speculated that the viral protein might be toxic to the hosts, killing the host once the protein was expressed. It is possible that these factors may play a role in inhibiting bacterial expression of the AHSV VP1 gene.

CHAPTER 5

CONCLUDING REMARKS

The recent breakthrough of the establishment of a system to recover infectious segmented negative-strand RNA viruses entirely from cDNA, and hence allow reverse genetics, has "profound implications for the study of viral life cycles and their regulation, the function of viral proteins, and the molecular mechanisms of viral pathogenicity" (Neumann *et al.* 1999). Unfortunately, no effective system has similarly yet been implemented to recover infectious multi-segmented dsRNA viruses from cDNA.

The objective of this study was to investigate VP1 of AHSV as the putative RNA-dependent RNA polymerase with a view to the potential future development of a reverse genetics system. In particular, the envisaged approach to this investigation involved the cloning and characterization, through sequencing, of the gene that encodes VP1 and the expression and activity analysis of the protein in a suitable eucaryotic expression system. The results have been discussed in detail in the relevant preceding chapters. These concluding remarks serve to highlight the pertinent information that contributes to a better understanding of the molecular biology of AHSV and points to future research in this field.

One of the bottlenecks which has long inhibited progress in the research of dsRNA viruses has been the cloning of genomic cDNA. In particular, it is notable that in the 18 years since the first description of the cloning of the dsRNA genes of reovirus (Cashdollar *et al.* 1982), and 10 years since the initial cloning of fragments of the genome segments of AHSV (Bremer *et al.* 1990), no full-length genome segment 1 gene, which encodes the putative viral RNA-dependent RNA polymerase, of any AHSV serotype has been cloned. Clearly, besides the obvious requirement of a complete complement of genomic clones for the establishment of a reverse genetics system, the central role of the RNA-dependent RNA polymerase in transcription and replication, and thus in the life cycle of the virus, makes this omission incisive.

An alternative method for the cloning of large dsRNA genome segments has been established. This approach involves ligation of an oligonucleotide containing convenient restriction enzyme recognition sequences and 3' extended with an oligo(dA) tail to the 3' termini of genomic dsRNA segments, followed by cDNA synthesis with an oligo(dT) primer and single primer PCR amplification. This technique was applied in the generation of a functional full-length AHSV VP1 gene during the course of this study, and subsequently extended to the cloning of complete genomes of multi-segmented dsRNA viruses (including rotavirus, EEV and AHSV) by other researchers in the laboratory (Potgieter, personal communication). This paves the way for further investigation and development of reverse genetics systems. In addition, the approach has also enabled the cloning of the genes encoding the major determinant of serotype-specificity and the neutralization-specific immune response (VP2) of all nine AHSV serotypes (Potgieter, personal communication), an important and long-awaited milestone in the pursuit of rapid diagnostics and effective subunit vaccines.

The nucleotide sequence of genome segment 2 dsRNA of AHSV-9 was determined, representing the first and, to date, only AHSV VP1 gene sequence published. Translation of the open reading frame enabled the identification of conserved RNA-dependent RNA polymerase-specific motifs and the definition of a signature for the polymerase sequences of the *Reoviridae*. Inter-serogroup comparisons of the RNA-dependent RNA polymerase amino acid sequences within the orbiviruses were carried out with previously published BTV and more recently published Chuzan virus sequences. The conservation exposed by these comparisons confirmed the limitations on sequence variability imposed by presumably functional specification. Grouping of the orbivirus serogroups could be clearly distinguished in phylogenetic analyses of the *Reoviridae* based on the RNA-dependent RNA polymerase amino acid sequences. However, distinction of the genera within the *Reoviridae* from other families proved less conspicuous. This corroborated previous arguments (Zanotto *et al.* 1996) that RNA-dependent RNA polymerase sequences cannot be used to construct a single phylogenetic tree including all RNA viruses, but that the evolutionary relationships between these viruses can more appropriately be presented as a set of distinct sub-trees, the links between which are unclear.

A further objective of this study was to express the RNA-dependent RNA polymerase of AHSV in a eucaryotic expression system and to assay for activity. Various systems for expression analysis were investigated, with varying levels of success. AHSV-9 VP1 was firstly expressed in a T7 RNA polymerase recombinant VACV system, whereby simultaneous synthesis of authentic mRNA sanctions *in vivo* replicase activity. Although expression of full-length protein was confirmed, no AHSV-specific RNA replication could be detected or confirmed. Unfortunately, circumstances inhibited further experimentation with this approach during the course of this study, but it is believed to have merit that requires further investigation.

AHSV VP1 was also expressed utilising the baculovirus system, yielding high levels of full-length protein. Although the expressed protein was insoluble, no attempts at solubilization, purification or assaying for polymerase activity were carried out. Precedents set with other viral RNA-dependent RNA polymerases expressed in insect cells demand investigation of AHSV VP1 activity. In addition, the recent reports of the formation of AHSV core-like particles in recombinant baculovirus-infected insect cells (Maree *et al.* 1998) invites investigation of protein-protein interactions between VP1 and core-like particles as well as other AHSV minor proteins. In the case of rotaviruses, protein-protein cross-linking studies have indicated that complexes of VP1 and NSP2 are present in rotavirus-infected cells (Kattoura *et al.* 1994). VP1 of rotaviruses has also been shown to bind specifically to *cis*-acting signals at the 3′ end of viral mRNA that serve to promote replication (Patton 1996) and that also include the entire target for the 3′-specific RNA-binding protein NSP3 (Poncet *et al.* 1994). Investigation and analysis of similar protein-protein and protein-RNA (ss- and dsRNA) interactions with expressed AHSV VP1 should improve our understanding of viral morphogenesis.

On the reverse genetics development front, additional research needs to address the gaps in our understanding of viral replication and morphogenesis. In theory, infectious dsRNA viral particles should be formed in cells in which a full complement of viral mRNA is introduced. However, the evidence suggests that the construction of active virus particles in infected cells is a complex but precise process, involving specific recruitment and localization of the components. As with the segmented ssRNA viruses, recovery of infectious reovirus from viral RNA required additional viral proteins, evidently specifically

through the formation of RNA-protein complexes (Roner *et al.* 1990). However, their function, the role of the helper virus and the mechanism by which dsRNA enhances the infectiousness of ssRNA are not clearly understood. In addition, the lack of subsequent reports confirms the complexity and questions the efficiency of this system.

Thus, a number of factors have to be taken into account in the development of a reverse genetics system for the dsRNA viruses. These include the RNA binding specificity of many viral proteins, the interactions between viral proteins and the accumulation of viral proteins and RNA at so-called virus inclusion bodies or viroplasms, which are believed to be sites for the early stages of virion assembly and morphogenesis.

The development of an effective system for the cloning of complete genomes of segmented dsRNA viruses, and the concomitant availability of a full complement of expressed viral proteins, including the RNA-dependent RNA polymerase, forms an excellent basis for ongoing research into the development of a reverse genetics system for AHSV, with potentially important implications.

REFERENCES

Antczak JB & Joklik WK (1992). Reovirus genome segment assortment into progeny genomes studied by the use of monoclonal antibodies directed against reovirus proteins. Virology *187*: 760-776.

Anzola JV, Xu ZK, Asamizu T & Nuss DL (1987). Segment-specific inverted repeats found adjacent to conserved terminal sequences in wound tumor virus genome and defective interfering RNAs. Proc.Natl.Acad.Sci.U.S.A *84*: 8301-8305.

Ball LA (1992). Cellular expression of a functional nodavirus RNA replicon from vaccinia virus vectors. J.Virol. *66*: 2335-2345.

Ball LA (1994). Replication of the genomic RNA of a positive-strand RNA animal virus from negative-sense transcripts. Proc.Natl.Acad.Sci.U.S.A *91*: 12443-12447.

Ball LA (1995). Requirements for the self-directed replication of flock house virus RNA 1 [published erratum appears in J Virol (1995) 69(4):2722]. J.Virol. *69*: 720-727.

Ball LA & Li Y (1993). cis-acting requirements for the replication of flock house virus RNA 2. J.Virol. *67*: 3544-3551.

Ball LA, Pringle CR, Flanagan B, Perepelitsa VP & Wertz GW (1999). Phenotypic consequences of rearranging the P, M, and G genes of vesicular stomatitis virus. J.Virol. *73*: 4705-4712.

Baltimore D, Huang AS & Stampfer M (1970). Ribonucleic acid synthesis of vesicular stomatitis virus, II. An RNA polymerase in the virion. Proc.Natl.Acad.Sci.U.S.A *66*: 572-576.

Bartlett NM, Gillies SC, Bullivant S & Bellamy AR (1974). Electron microscopy study of reovirus reaction cores. J.Virol. *14*: 315-326.

Baudin F, Bach C, Cusack S & Ruigrok RW (1994). Structure of influenza virus RNP. I. Influenza virus nucleoprotein melts secondary structure in panhandle RNA and exposes the bases to the solvent. EMBO J. *13*: 3158-3165.

Bigot Y, Drezen JM, Sizaret PY, Rabouille A, Hamelin MH & Periquet G (1995). The genome segments of DpRV, a commensal reovirus of the wasp Diadromus pulchellus (Hymenoptera). Virology *210*: 109-119.

Both GW, Bellamy AR, Street JE & Siegman LJ (1982). A general strategy for cloning double-stranded RNA: nucleotide sequence of the Simian-11 rotavirus gene 8. Nucleic Acids Res. *10*: 7075-7088.

Boyer JC & Haenni AL (1994). Infectious transcripts and cDNA clones of RNA viruses. Virology *198*: 415-426.

Bremer CW, Huismans H & Van Dijk AA (1990). Characterization and cloning of the African horsesickness virus genome. J.Gen.Virol. *71 (Pt 4)*: 793-799.

Bridgen A & Elliott RM (1996). Rescue of a segmented negative-strand RNA virus entirely from cloned complementary DNAs. Proc.Natl.Acad.Sci.U.S.A *93*: 15400-15404.

Brown EG, Nibert ML & Fields BN (1983). The L2 gene of reovirus serotype 3 controls the capacity to interfere, accumulate deletions and establish persistent infection. *In* Double-stranded RNA viruses. Compans RW & Bishop DHL (Eds.). Elsevier: New York.

Bruenn JA (1991). Relationships among the positive strand and double-strand RNA viruses as viewed through their RNA-dependent RNA polymerases. Nucleic Acids Res. *19*: 217-226.

Bruenn JA (1993). A closely related group of RNA-dependent RNA polymerases from double- stranded RNA viruses. Nucleic Acids Res. *21*: 5667-5669.

Buck KW (1996). Comparison of the replication of positive-stranded RNA viruses of plants and animals. Adv.Virus Res. *47*: 159-251.

Cashdollar LW, Esparza J, Hudson GR, Chmelo R, Lee PW & Joklik WK (1982). Cloning the double-stranded RNA genes of reovirus: sequence of the cloned S2 gene. Proc.Natl.Acad.Sci.U.S.A *79*: 7644-7648.

Cashdollar LW, Chmelo R, Esparza J, Hudson GR & Joklik WK (1984). Molecular cloning of the complete genome of reovirus serotype 3. Virology *133*: 191-196.

Cech TR (1986). RNA as an enzyme. Sci.Am. *255*: 64-75.

Chen D & Patton JT (1998). Rotavirus RNA replication requires a single-stranded 3' end for efficient minus-strand synthesis. J.Virol. *72*: 7387-7396.

Chen B, Choi GH & Nuss DL (1994a). Attenuation of fungal virulence by synthetic infectious hypovirus transcripts. Science *264*: 1762-1764.

Chen D, Zeng CQ, Wentz MJ, Gorziglia M, Estes MK & Ramig RF (1994b). Template-dependent, in vitro replication of rotavirus RNA. J.Virol. *68*: 7030-7039.

Cline J, Braman JC & Hogrefe HH (1996). PCR fidelity of pfu DNA polymerase and other thermostable DNA polymerases. Nucleic Acids Res. *24*: 3546-3551.

Cohen J (1977). Ribonucleic acid polymerase activity associated with purified calf rotavirus. J.Gen.Virol. *36*: 395-402.

Cohen J, Charpilienne A, Chilmonczyk S & Estes MK (1989a). Nucleotide sequence of bovine rotavirus gene 1 and expression of the gene product in baculovirus. Virology *171*: 131-140.

Cohen JI, Wang F, Mannick J & Kieff E (1989b). Epstein-Barr virus nuclear protein 2 is a key determinant of lymphocyte transformation. Proc.Natl.Acad.Sci.U.S.A *86*: 9558-9562.

Collins PL, Mink MA & Stec DS (1991). Rescue of synthetic analogs of respiratory syncytial virus genomic RNA and effect of truncations and mutations on the expression of a foreign reporter gene. Proc.Natl.Acad.Sci.U.S.A *88*: 9663-9667.

Collins PL, Hill MG, Camargo E, Grosfeld H, Chanock RM & Murphy BR (1995). Production of infectious human respiratory syncytial virus from cloned cDNA confirms an essential role for the transcription elongation factor from the 5' proximal open reading frame of the M2 mRNA in gene expression and provides a capability for vaccine development. Proc.Natl.Acad.Sci.U.S.A *92*: 11563-11567.

Cooke SJ, Lambden PR, Caul EO & Clarke IN (1991). Molecular cloning, sequence analysis and coding assignment of the major inner capsid protein gene of human group C rotavirus. Virology *184*: 781-785.

Coombs KM (1998). Stoichiometry of reovirus structural proteins in virus, ISVP, and core particles. Virology *243*: 218-228.

Cunningham C & Davison AJ (1993). A cosmid-based system for constructing mutants of herpes simplex virus type 1. Virology *197*: 116-124.

DePolo NJ, Giachetti C & Holland JJ (1987). Continuing coevolution of virus and defective interfering particles and of viral genome sequences during undiluted passages: virus mutants exhibiting nearly complete resistance to formerly dominant defective interfering particles. J.Virol. *61*: 454-464.

Devi R & Roy P (1999). Polymerase complex of Bluetongue virus. *In* Conference Proceedings: XIth International Congress of Virology. 9-13 August. Sydney, Australia.

Dryden KA, Wang G, Yeager M, Nibert ML, Coombs KM, Furlong DB, Fields BN & Baker TS (1993). Early steps in reovirus infection are associated with dramatic changes in supramolecular structure and protein conformation: analysis of virions and subviral particles by cryoelectron microscopy and image reconstruction. J.Cell Biol. *122*: 1023-1041.

Dryden KA, Farsetta DL, Wang G, Keegan JM, Fields BN, Baker TS & Nibert ML (1998). Internal/structures containing transcriptase-related proteins in top component particles of mammalian orthoreovirus. Virology *245*: 33-46.

Dzianott AM & Bujarski JJ (1988). An in vitro transcription vector which generates nearly correctly ended RNAs by self-cleavage of longer transcripts. Nucleic Acids Res. *16*: 10940

Eckert KA & Kunkel TA (1991). DNA polymerase fidelity and the polymerase chain reaction. PCR Methods Appl. *1*: 17-24.

Emerson SU & Wagner RR (1972). Dissociation and reconstitution of the transcriptase and template activities of vesicular stomatitis B and T virions. J.Virol. *10*: 297-309.

Enami M, Luytjes W, Krystal M & Palese P (1990). Introduction of site-specific mutations into the genome of influenza virus. Proc.Natl.Acad.Sci.U.S.A *87*: 3802-3805.

Enami M, Sharma G, Benham C & Palese P (1991). An influenza virus containing nine different RNA segments [published erratum appears in Virology (1992) 186(2):798]. Virology *185*: 291-298.

Fields BN (1996). Fields Virology. Fields BN, Knipe DM & Howley PM (Eds.). Lippincott-Raven Publishers: Philadelphia.

Finke S & Conzelmann KK (1999). Virus promoters determine interference by defective RNAs: selective amplification of mini-RNA vectors and rescue from cDNA by a 3' copy- back ambisense rabies virus. J.Virol. *73*: 3818-3825.

Fodor E, Devenish L, Engelhardt OG, Palese P, Brownlee GG & Garcia-Sastre A (1999). Rescue of influenza A virus from recombinant DNA. J.Virol. *73*: 9679-9682.

French TJ & Roy P (1990). Synthesis of bluetongue virus (BTV) corelike particles by a recombinant baculovirus expressing the two major structural core proteins of BTV. J.Virol. *64*: 1530-1536.

French TJ, Marshall JJ & Roy P (1990). Assembly of double-shelled, viruslike particles of bluetongue virus by the simultaneous expression of four structural proteins. J.Virol. *64*: 5695-5700.

Fuerst TR, Niles EG, Studier FW & Moss B (1986). Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc.Natl.Acad.Sci.U.S.A *83*: 8122-8126.

Fukusho A, Yu Y, Yamaguchi S & Roy P (1989). Completion of the sequence of bluetongue virus serotype 10 by the characterization of a structural protein, VP6, and a non-structural protein, NS2. J.Gen.Virol. *70 (Pt 7)*: 1677-1689.

Galili G, Kawata EE, Cuellar RE, Smith LD & Larkins BA (1986). Synthetic oligonucleotide tails inhibit in vitro and in vivo translation of SP6 transcripts of maize zein cDNA clones. Nucleic Acids Res. *14*: 1511-1524.

Gallegos CO & Patton JT (1989). Characterization of rotavirus replication intermediates: a model for the assembly of single-shelled particles. Virology *172*: 616-627.

Genetics Computer Group (1994). Program manual for the Wisconsin package [Version 8]. 575 Science Drive, Madison, Wisconsin, USA 53711.

Goff SP & Berg P (1976). Construction of hybrid viruses containing SV40 and lambda phage DNA segments and their propagation in cultured monkey cells. Cell *9*: 695-705.

Gombold JL, Estes MK & Ramig RF (1985). Assignment of simian rotavirus SA11 temperature-sensitive mutant groups B and E to genome segments. Virology *143*: 309-320.

Gottlieb P, Strassman J, Bamford DH & Mindich L (1988). Production of a polyhedral particle in Escherichia coli from a cDNA copy of the large genomic segment of bacteriophage phi 6. J.Virol. *62*: 181-187.

Gottlieb P, Strassman J, Qiao XY, Frucht A & Mindich L (1990). In vitro replication, packaging, and transcription of the segmented double-stranded RNA genome of bacteriophage phi 6: studies with procapsids assembled from plasmid-encoded proteins. J.Bacteriol. *172*: 5774-5782.

Gouet P, Diprose JM, Grimes JM, Malby R, Burroughs JN, Zientara S, Stuart DI & Mertens PP (1999). The highly ordered double-stranded RNA genome of bluetongue virus revealed by crystallography. Cell *97*: 481-490.

Grimes JM, Burroughs JN, Gouet P, Diprose JM, Malby R, Zientara S, Mertens PP & Stuart DI (1998). The atomic structure of the bluetongue virus core. Nature *395*: 470-478.

Habili N & Symons RH (1989). Evolutionary relationship between luteoviruses and other RNA plant viruses based on sequence motifs in their putative RNA polymerases and nucleic acid helicases. Nucleic Acids Res. *17*: 9543-9555.

Hansen JL, Long AM & Schultz SC (1997). Structure of the RNA-dependent RNA polymerase of poliovirus. Structure. *5*: 1109-1122.

Hruby DE, Guarino LA & Kates JR (1979). Vaccinia virus replication. I. Requirement for the host-cell nucleus. J.Virol. *29*: 705-715.

Huang IJ, Hwang GY, Yang YY, Hayama E & Li JK (1995). Sequence analyses and antigenic epitope mapping of the putative RNA- directed RNA polymerase of five U.S. bluetongue viruses. Virology *214*: 280-288.

Huismans H & Verwoerd DW (1973). Control of transcription during the expression of the bluetongue virus genome. Virology *52*: 81-88.

Huismans H & Cloete M (1987). A comparison of different cloned bluetongue virus genome segments as probes for the detection of virus-specified RNA. Virology *158*: 373-380.

Huismans H, Bremer CW & Barber TL (1979). The nucleic acid and proteins of epizootic haemorrhagic disease virus. Onderstepoort J.Vet.Res. *46*: 95-104.

Hwang GY & Li JK (1993). Identification and localization of a serotypic neutralization determinant on the VP2 protein of bluetongue virus 13. Virology *195*: 859-862.

Hwang GY, Xiang M & Li JK (1994). Analyses and conservation of sequences among the cognate L3 segments of the five United States bluetongue viruses. Virus Res. *32*: 381-389.

Johnson KL & Ball LA (1997). Replication of flock house virus RNAs from primary transcripts made in cells by RNA polymerase II. J.Virol. *71*: 3323-3327.

Joklik WK (1983). The reovirus particle. *In* The Reoviridae. Joklik WK (Eds.). Plenum Press: New York, pp 9-78.

Joklik WK (1998). Assembly of the reovirus genome. Curr.Top.Microbiol.Immunol. *233 REOVIR.I*: 57-68.

Joklik WK & Roner MR (1995). What reassorts when reovirus genome segments reassort? J.Biol.Chem. *270*: 4181-4184.

Joklik WK & Roner MR (1996). Molecular recognition in the assembly of the segmented reovirus genome. Prog.Nucleic Acid Res.Mol.Biol. *53*: 249-281.

Jones N & Shenk T (1978). Isolation of deletion and substitution mutants of adenovirus type 5. Cell *13*: 181-188.

Kamer G & Argos P (1984). Primary structural comparison of RNA-dependent polymerases from plant, animal and bacterial viruses. Nucleic Acids Res. *12*: 7269-7282.

Kapuler AM (1970). An extraordinary temperature dependence of the reovirus transcriptase. Biochemistry *9*: 4453-4457.

Kattoura MD, Clapp LL & Patton JT (1992). The rotavirus nonstructural protein, NS35, possesses RNA-binding activity in vitro and in vivo. Virology *191*: 698-708.

Kattoura MD, Chen X & Patton JT (1994). The rotavirus RNA-binding protein NS35 (NSP2) forms 10S multimers and interacts with the viral RNA polymerase. Virology *202*: 803-813.

Kemble G, Duke G, Winter R & Spaete R (1996). Defined large-scale alterations of the human cytomegalovirus genome constructed by cotransfection of overlapping cosmids. J.Virol. *70*: 2044-2048.

Kumar S, Tamura K & Nei M (1994). MEGA: Molecular Evolutionary Genetics Analysis software for microcomputers. Comput. Appl. Biosci. *10*: 189-191.

Koonin EV (1991). The phylogeny of RNA-dependent RNA polymerases of positive-strand RNA viruses. J.Gen.Virol. *72 (Pt 9)*: 2197-2206.

Koonin EV & Dolja VV (1993). Evolution and taxonomy of positive-strand RNA viruses: implications of comparative analysis of amino acid sequences [published erratum appears in Crit Rev Biochem Mol Biol (1993) 28(6):546]. Crit Rev.Biochem.Mol.Biol. *28*: 375-430.

Koonin EV, Gorbalenya AE & Chumakov KM (1989). Tentative identification of RNA-dependent RNA polymerases of dsRNA viruses and their relationship to positive strand RNA viral polymerases. FEBS Lett. *252*: 42-46.

Kowalik TF, Yang YY & Li JK (1990). Molecular cloning and comparative sequence analyses of bluetongue virus S1 segments by selective synthesis of specific full-length DNA copies of dsRNA genes. Virology *177*: 820-823.

Kozak M (1981). Possible role of flanking nucleotides in recognition of the AUG initiator codon by eukaryotic ribosomes. Nucleic Acids Res. *9*: 5233-5262.

Kozak M (1984). Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. Nucleic Acids Res. *12*: 857-872.

Kyte J & Doolittle RF (1982). A simple method for displaying the hydropathic character of a protein. J.Mol.Biol. *157*: 105-132.

Lai VC, Kao CC, Ferrari E, Park J, Uss AS, Wright-Minogue J, Hong Z & Lau JY (1999). Mutational analysis of bovine viral diarrhea virus RNA-dependent RNA polymerase. J.Virol. *73*: 10129-10136.

Lamb RA & Choppin PW (1983). The gene structure and replication of influenza virus. Annu.Rev.Biochem. *52*: 467-506.

Lambden PR, Cooke SJ, Caul EO & Clarke IN (1992). Cloning of noncultivatable human rotavirus by single primer amplification. J.Virol. *66*: 1817-1822.

Larson SM, Antczak JB & Joklik WK (1994). Reovirus exists in the form of 13 particle species that differ in their content of protein sigma 1. Virology *201*: 303-311.

Lawson ND, Stillman EA, Whitt MA & Rose JK (1995). Recombinant vesicular stomatitis viruses from DNA [published erratum appears in Proc Natl Acad Sci U S A (1995) 92(19):9009]. Proc.Natl.Acad.Sci.U.S.A *92*: 4477-4481.

Le Blois H, Fayard B, Urakawa T & Roy P (1991). Synthesis and characterization of chimeric particles between epizootic hemorrhagic disease virus and bluetongue virus: functional domains are conserved on the VP3 protein. J.Virol. *65*: 4821-4831.

Le Blois H, French T, Mertens PP, Burroughs JN & Roy P (1992). The expressed VP4 protein of bluetongue virus binds GTP and is the candidate guanylyl transferase of the virus. Virology *189*: 757-761.

Lehrach H, Diamond D, Wozney JM & Boedtker H (1977). RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. Biochemistry *16*: 4743-4751.

Levis R, Weiss BG, Tsiang M, Huang H & Schlesinger S (1986). Deletion mapping of Sindbis virus DI RNAs derived from cDNAs defines the sequences essential for replication and packaging. Cell *44*: 137-145.

Li YI, Cheng YM, Huang YL, Tsai CH, Hsu YH & Meng M (1998). Identification and characterization of the Escherichia coli-expressed RNA-dependent RNA polymerase of bamboo mosaic virus. J.Virol. *72*: 10093-10099.

Liljestrom P, Lusa S, Huylebroeck D & Garoff H (1991). In vitro mutagenesis of a full-length cDNA clone of Semliki Forest virus: the small 6,000-molecular-weight membrane protein modulates virus release. J.Virol. *65*: 4107-4113.

Ling ML, Risman SS, Klement JF, McGraw N & McAllister WT (1989). Abortive initiation by bacteriophage T3 and T7 RNA polymerases under conditions of limiting substrate [published erratum appears in Nucleic Acids Res (1989) 17(11):4430]. Nucleic Acids Res. *17*: 1605-1618.

Loening UE (1967). The fractionation of high-molecular-weight ribonucleic acid by polyacrylamide-gel electrophoresis. Biochem.J. *102*: 251-257.

Long DM & Uhlenbeck OC (1993). Self-cleaving catalytic RNA. FASEB J. *7*: 25-30.

Loudon PT & Roy P (1991). Assembly of five bluetongue virus proteins expressed by recombinant baculoviruses: inclusion of the largest protein VP1 in the core and virus-like proteins. Virology *180*: 798-802.

Loudon PT & Roy P (1992). Interaction of nucleic acids with core-like and subcore-like particles of bluetongue virus. Virology *191*: 231-236.

Luckow VA, Lee SC, Barry GF & Olins PO (1993). Efficient generation of infectious recombinant baculoviruses by site- specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in Escherichia coli. J.Virol. *67*: 4566-4579.

Mackett M, Smith GL & Moss B (1982). Vaccinia virus: a selectable eukaryotic cloning and expression vector. Proc.Natl.Acad.Sci.U.S.A *79*: 7415-7419.

Makeyev EV & Bamford DH (2000). Replicase activity of purified recombinant protein P2 of double- stranded RNA bacteriophage phi6. EMBO J. *19*: 124-133.

Makino S, Yokomori K & Lai MM (1990). Analysis of efficiently packaged defective interfering RNAs of murine coronavirus: localization of a possible RNA-packaging signal. J.Virol. *64*: 6045-6053.

Maree S, Durbach S & Huismans H (1998). Intracellular production of African horsesickness virus core-like particles by expression of the two major core proteins, VP3 and VP7, in insect cells. J.Gen.Virol. *79 (Pt 2)*: 333-337.

Mebatsion T & Conzelmann KK (1996). Specific infection of CD4+ target cells by recombinant rabies virus pseudotypes carrying the HIV-1 envelope spike protein. Proc.Natl.Acad.Sci.U.S.A *93*: 11366-11370.

Mebatsion T, Schnell MJ, Cox JH, Finke S & Conzelmann KK (1996). Highly stable expression of a foreign gene from rabies virus vectors. Proc.Natl.Acad.Sci.U.S.A *93*: 7310-7314.

Mertens PP, Jamieson PB & Dobos P (1982). In vitro RNA synthesis by infectious pancreatic necrosis virus- associated RNA polymerase. J.Gen.Virol. *59*: 47-56.

Mindich L (1999). Precise packaging of the three genomic segments of the double-stranded- RNA bacteriophage phi6. Microbiol.Mol.Biol.Rev. *63*: 149-160.

Mitchell DB & Both GW (1990). Completion of the genomic sequence of the simian rotavirus SA11: nucleotide sequences of segments 1, 2, and 3. Virology *177*: 324-331.

Mizukoshi N, Sakamoto K, Iwata A, Tsuchiya T, Ueda S, Apiwatnakorn B, Kamada M & Fukusho A (1993). The complete nucleotide sequence of African horsesickness virus serotype 4 (vaccine strain) segment 4, which encodes the minor core protein VP4. Virus Res. *28*: 299-306.

Moody MD & Joklik WK (1989). The function of reovirus proteins during the reovirus multiplication cycle: analysis using monoreassortants. Virology *173*: 437-446.

Morozov SY (1989). A possible relationship of reovirus putative RNA polymerase to polymerases of positive-strand RNA viruses. Nucleic Acids Res. *17*: 5394

Mulligan RC (1993). The basic science of gene therapy. Science *260*: 926-932.

Mundt E & Vakharia VN (1996). Synthetic transcripts of double-stranded Birnavirus genome are infectious. Proc.Natl.Acad.Sci.U.S.A *93*: 11131-11136.

Murphy FA, Fauquet CM, Bishop DHL, Ghabrial SA, Jarvis AW, Martelli GP, Mayo MA & Summers MD (1995). Virus Taxonomy: The Classification and Nomenclature of Viruses. The Sixth Report of the International Committee on Taxonomy of Viruses. Springer-Verlag: Vienna.

Nagai Y (1999). Paramyxovirus replication and pathogenesis. Reverse genetics transforms understanding. Rev.Med.Virol. *9*: 83-99.

Nakashima N, Koizumi M, Watanabe H & Noda H (1996). Complete nucleotide sequence of the Nilaparvata lugens reovirus: a putative member of the genus Fijivirus. J.Gen.Virol. *77 (Pt 1)*: 139-146.

Namiki M, Suzuki Y, Nakagawa S, Yoshioka I & Saito Y (1983). Characterization of ribonucleic acid transcriptase in Ibaraki virus core particles. J.Gen.Virol. *64 (Pt 6)*: 1397-1400.

Nel LH & Huismans H (1991). Synthesis of the virus-specified tubules of epizootic haemorrhagic disease virus using a baculovirus expression system. Virus Res. *19*: 139-152.

Neumann G, Watanabe T, Ito H, Watanabe S, Goto H, Gao P, Hughes M, Perez DR, Donis R, Hoffmann E, Hobom G & Kawaoka Y (1999). Generation of influenza A viruses entirely from cloned cDNAs [see comments]. Proc.Natl.Acad.Sci.U.S.A *96*: 9345-9350.

O'Reilly EK & Kao CC (1998). Analysis of RNA-dependent RNA polymerase structure and function as guided by known polymerase structures and computer predictions of secondary structure. Virology *252*: 287-303.

O'Reilly DR, Miller LK & Luckow VA (1992). Baculovirus expression vectors: A laboratory manual. W.H. Freeman and Company: New York.

Olkkonen VM, Gottlieb P, Strassman J, Qiao XY, Bamford DH & Mindich L (1990). In vitro assembly of infectious nucleocapsids of bacteriophage phi 6: formation of a recombinant double-stranded RNA virus. Proc.Natl.Acad.Sci.U.S.A *87*: 9173-9177.

Panicali D & Paoletti E (1982). Construction of poxviruses as cloning vectors: insertion of the thymidine kinase gene from herpes simplex virus into the DNA of infectious vaccinia virus. Proc.Natl.Acad.Sci.U.S.A *79*: 4927-4931.

Park KH, Huang T, Correia FF & Krystal M (1991). Rescue of a foreign gene by Sendai virus. Proc.Natl.Acad.Sci.U.S.A *88*: 5537-5541.

Pattnaik AK, Ball LA, LeGrone AW & Wertz GW (1992). Infectious defective interfering particles of VSV from transcripts of a cDNA clone. Cell *69*: 1011-1020.

Patton JT (1990). Evidence for equimolar synthesis of double-strand RNA and minus-strand RNA in rotavirus-infected cells. Virus Res. *17*: 199-208.

Patton JT (1996). Rotavirus VP1 alone specifically binds to the 3' end of viral mRNA, but the interaction is not sufficient to initiate minus-strand synthesis. J.Virol. *70*: 7940-7947.

Patton JT & Gallegos CO (1990). Rotavirus RNA replication: single-stranded RNA extends from the replicase particle. J.Gen.Virol. *71 (Pt 5)*: 1087-1094.

Patton JT, Jones MT, Kalbach AN, He YW & Xiaobo J (1997). Rotavirus RNA polymerase requires the core shell protein to synthesize the double-stranded RNA genome. J.Virol. *71*: 9618-9626.

Patton JT, Chnaiderman J & Spencer E (1999). Open reading frame in rotavirus mRNA specifically promotes synthesis of double-stranded RNA: template size also affects replication efficiency. Virology *264*: 167-180.

Perrotta AT & Been MD (1991). A pseudoknot-like structure required for efficient self-cleavage of hepatitis delta virus RNA. Nature *350*: 434-436.

Pleschka S, Jaskunas R, Engelhardt OG, Zurcher T, Palese P & Garcia-Sastre A (1996). A plasmid-based reverse genetics system for influenza A virus. J.Virol. *70*: 4188-4192.

Ploegh HL (1998). Viral strategies of immune evasion. Science *280*: 248-253.

Poch O, Sauvaget I, Delarue M & Tordo N (1989). Identification of four conserved motifs among the RNA-dependent polymerase encoding elements. EMBO J. *8*: 3867-3874.

Poncet D, Laurent S & Cohen J (1994). Four nucleotides are the minimal requirement for RNA recognition by rotavirus non-structural protein NSP3. EMBO J. *13*:4165-4173.

Poranen MM & Bamford DH (1999). Packaging and replication regulation revealed by chimeric genome segments of double-stranded RNA bacteriophage phi6. RNA. *5*: 446-454.

Post LE & Roizman B (1981). A generalized technique for deletion of specific genes in large genomes: alpha gene 22 of herpes simplex virus 1 is not essential for growth. Cell *25*: 227-232.

Potgieter AC. Onderstepoort Veterinary Institute, Private Bag X5, Onderstepoort 0110, South Africa.

Prasad BV, Wang GJ, Clerx JP & Chiu W (1988). Three-dimensional structure of rotavirus. J.Mol.Biol. *199*: 269-275.

Purdy M, Petre J & Roy P (1984). Cloning of the bluetongue virus L3 gene. J.Virol. *51*: 754-759.

Racaniello VR & Baltimore D (1981). Cloned poliovirus complementary DNA is infectious in mammalian cells. Science *214*: 916-919.

Radecke F, Spielhofer P, Schneider H, Kaelin K, Huber M, Dotsch C, Christiansen G & Billeter MA (1995). Rescue of measles viruses from cloned DNA. EMBO J. *14*: 5773-5784.

Rice CM, Levis R, Strauss JH & Huang HV (1987). Production of infectious RNA transcripts from Sindbis virus cDNA clones: mapping of lethal mutations, rescue of a temperature-sensitive marker, and in vitro mutagenesis to generate defined mutants. J.Virol. *61*: 3809-3819.

Roner MR, Sutphin LA & Joklik WK (1990). Reovirus RNA is infectious. Virology *179*: 845-852.

Roner MR, Lin PN, Nepluev I, Kong LJ & Joklik WK (1995). Identification of signals required for the insertion of heterologous genome segments into the reovirus genome. Proc.Natl.Acad.Sci.U.S.A *92*: 12362-12366.

Rost B & Sander C (1994). Combining evolutionary information and neural networks to predict protein secondary structure. Proteins *19*: 55-72.

Roy P (1992). Bluetongue virus proteins. J.Gen.Virol. *73 (Pt 12)*: 3051-3064.

Roy P (1996). Orbivirus structure and assembly [published erratum appears in Virology (1996) 218(1):296]. Virology *216*: 1-11.

Roy P, Fukusho A, Ritter GD & Lyon D (1988). Evidence for genetic relationship between RNA and DNA viruses from the sequence homology of a putative polymerase gene of bluetongue virus with that of vaccinia virus: conservation of RNA polymerase genes from diverse species. Nucleic Acids Res. *16*: 11759-11767.

Roy P, Mertens PP & Casal I (1994). African horse sickness virus structure. Comp Immunol.Microbiol.Infect.Dis. *17*: 243-273.

Sakamoto K, Mizukoshi N, Apiwatnakorn B, Iwata A, Tsuchiya T, Ueda S, Imagawa H, Sugiura T, Kamada M & Fukusho A (1994). The complete sequences of African horsesickness virus serotype 4 (vaccine strain) RNA segment 2 and 6 which encode outer capsid protein. J.Vet.Med.Sci. *56*: 321-327.

Sambrook J, Fritsch EF & Maniatis T (1989). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York.

Samulski RJ, Chang LS & Shenk T (1989). Helper-free stocks of recombinant adeno-associated viruses: normal integration does not require viral gene expression. J.Virol. *63*: 3822-3828.

Sanger F, Nicklen S & Coulson AR (1977). DNA sequencing with chain-terminating inhibitors. Proc.Natl.Acad.Sci.U.S.A *74*: 5463-5467.

Schneider H, Spielhofer P, Kaelin K, Dotsch C, Radecke F, Sutter G & Billeter MA (1997). Rescue of measles virus using a replication-deficient vaccinia-T7 vector. J.Virol.Methods *64*: 57-64.

Schnell MJ, Mebatsion T & Conzelmann KK (1994). Infectious rabies viruses from cloned cDNA. EMBO J. *13*: 4195-4203.

Shapouri MR, Kane M, Letarte M, Bergeron J, Arella M & Silim A (1995). Cloning, sequencing and expression of the S1 gene of avian reovirus. J.Gen.Virol. *76 (Pt 6)*: 1515-1520.

Shatkin AJ & Kozak M (1983). Biochemical aspects of reovirus transcription and translation. *In* The Reoviridae. Joklik WK (Eds.). Plenum Press: New York, pp 79-106.

Sidhu MS, Chan J, Kaelin K, Spielhofer P, Radecke F, Schneider H, Masurekar M, Dowling PC, Billeter MA & Udem SA (1995). Rescue of synthetic measles virus minireplicons: measles genomic termini direct efficient expression and propagation of a reporter gene. Virology *208* : 800-807.

Silber R, Malathi VG & Hurwitz J (1972). Purification and properties of bacteriophage T4-induced RNA ligase. Proc.Natl.Acad.Sci.U.S.A *69*: 3009-3013.

Snopek TJ, Sugino A, Agarwal KL & Cozzarelli NR (1976). Catalysis of DNA joining by bacteriophage T4 RNA ligase. Biochem.Biophys.Res.Commun. *68*: 417-424.

Sosnovtsev S & Green KY (1995). RNA transcripts derived from a cloned full-length copy of the feline calicivirus genome do not require VpG for infectivity. Virology *210*: 383-390.

Spendlove RS, McClain ME & Lennette EH (1970). Enhancement of reovirus infectivity by extracellular removal or alteration of the virus capsid by proteolytic enzymes. J.Gen.Virol. *8*: 83-94.

Spies U, Muller H & Becht H (1987). Properties of RNA polymerase activity associated with infectious bursal disease virus and characterization of its reaction products. Virus Res. *8*: 127-140.

Starnes MC & Joklik WK (1993). Reovirus protein lambda 3 is a poly(C)-dependent poly(G) polymerase. Virology *193*: 356-366.

Summers MD & Smith GE (1987). A manual of methods for baculovirus vectors and insect cell culture procedures. Texan Agric.Exp.Station Bull. *No 1555*.

Suzuki N, Tanimura M, Watanabe Y, Kusano T, Kitagawa Y, Suda N, Kudo H, Uyeda I & Shikata E (1992). Molecular analysis of rice dwarf phytoreovirus segment S1: interviral homology of the putative RNA-dependent RNA polymerase between plant- and animal-infecting reoviruses. Virology *190*: 240-247.

Tessier DC, Brousseau R & Vernet T (1986). Ligation of single-stranded oligodeoxyribonucleotides by T4 RNA ligase. Anal.Biochem. *158*: 171-178.

Thomas CP, Booth TF & Roy P (1990). Synthesis of bluetongue virus-encoded phosphoprotein and formation of inclusion bodies by recombinant baculovirus in insect cells: it binds the single-stranded RNA species. J.Gen.Virol. *71 (Pt 9)*: 2073-2083.

Thomson JM & Parrott WA (1998). pMECA: a cloning plasmid with 44 unique restriction sites that allows selection of recombinants based on colony size. Biotechniques *24*: 922-4, 926, 928.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F & Higgins DG (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. *25*: 4876-4882.

Turnbull PJ, Cormack SB & Huismans H (1996). Characterization of the gene encoding core protein VP6 of two African horsesickness virus serotypes. J.Gen.Virol. *77 (Pt 7)*: 1421-1423.

Upadhyaya NM, Ramm K, Gellatly JA, Li Z, Kositratana W & Waterhouse PM (1998). Rice ragged stunt oryzavirus genome segment S4 could encode an RNA dependent RNA polymerase and a second protein of unknown function. Arch.Virol. *143*: 1815-1822.

Urakawa T, Ritter DG & Roy P (1989). Expression of largest RNA segment and synthesis of VP1 protein of bluetongue virus in insect cells by recombinant baculovirus: association of VP1 protein with RNA polymerase activity. Nucleic Acids Res. *17*: 7395-7401.

Uyeda I, Kimura I & Shikata E (1995). Characterization of genome structure and establishment of vector cell lines for plant reoviruses. Adv.Virus Res. *45*: 249-279.

Van der Most RG, Bredenbeek PJ & Spaan WJ (1991). A domain at the 3' end of the polymerase gene is essential for encapsidation of coronavirus defective interfering RNAs. J.Virol. *65*: 3219-3226.

Van Dijk AA & Huismans H (1980). The in vitro activation and further characterization of the bluetongue virus-associated transcriptase. Virology *104*: 347-356.

Van Dijk AA & Huismans H (1982). The effect of temperature on the in vitro transcriptase reaction of bluetongue virus, epizootic haemorrhagic disease virus and African horsesickness virus. Onderstepoort J.Vet.Res. *49*: 227-232.

Van Dijk AA & Huismans H (1988). In vitro transcription and translation of bluetongue virus mRNA. J.Gen.Virol. *69 (Pt 3)*: 573-581.

Van Staden V, Theron J, Greyling BJ, Huismans H & Nel LH (1991). A comparison of the nucleotide sequences of cognate NS2 genes of three different orbiviruses. Virology *185*: 500-504.

Vende P, Piron M, Castagne N & Poncet D (2000). Efficient translation of rotavirus mRNA requires simultaneous interaction of NSP3 with the eukaryotic translation initiation factor eIF4G and the mRNA 3' End. J.Virol. *74*: 7064-7071.

Verwoerd DW & Huismans H (1972). Studies on the in vitro and the in vivo transcription of the bluetongue virus genome. Onderstepoort J.Vet.Res. *39*: 185-191.

Viljoen GJ & Cloete MC. Onderstepoort Veterinary Institute, Private Bag X5. Onderstepoort 0110, South Africa.

Vreede FT (1994). Cloning, characterization and expression of the gene that encodes the major neutralization-specific antigen of African horsesickness virus. University of Pretoria (*MSc Thesis/Dissertation*).

Wei CM, Gibson M, Spear PG & Scolnick EM (1981). Construction and isolation of a transmissible retrovirus containing the src gene of Harvey murine sarcoma virus and the thymidine kinase gene of herpes simplex virus type 1. J.Virol. *39*: 935-944.

Wentz MJ, Patton JT & Ramig RF (1996). The 3'-terminal consensus sequence of rotavirus mRNA is the minimal promoter of negative-strand RNA synthesis. J.Virol. *70*: 7833-7841.

Wertz GW, Perepelitsa VP & Ball LA (1998). Gene rearrangement attenuates expression and lethality of a nonsegmented negative strand RNA virus. Proc.Natl.Acad.Sci.U.S.A *95*: 3501-3506.

Whelan SP, Ball LA, Barr JN & Wertz GT (1995). Efficient recovery of infectious vesicular stomatitis virus entirely from cDNA clones. Proc.Natl.Acad.Sci.U.S.A *92*: 8388-8392.

Wiener JR & Joklik WK (1989). The sequences of the reovirus serotype 1, 2, and 3 L1 genome segments and analysis of the mode of divergence of the reovirus serotypes. Virology *169*: 194-203.

Yamakawa M, Furuichi Y, Nakashima K, LaFiandra AJ & Shatkin AJ (1981). Excess synthesis of viral mRNA 5-terminal oligonucleotides by reovirus transcriptase. J.Biol.Chem. *256*: 6507-6514.

Yamakawa M, Furuichi Y & Shatkin AJ (1982). Reovirus transcriptase and capping enzymes are active in intact virions. Virology *118*: 157-168.

Yamakawa M, Furuuchi S & Minobe Y (1999a). Molecular characterization of double-stranded RNA segments encoding the major capsid proteins of a Palyam serogroup orbivirus that caused an epizootic of congenital abnormalities in cattle. J.Gen.Virol. *80 (Pt 1)*: 205-208.

Yamakawa M, Kubo M & Furuuchi S (1999b). Molecular analysis of the genome of Chuzan virus, a member of the Palyam serogroup viruses, and its phylogenetic relationships to other orbiviruses. J.Gen.Virol. *80 (Pt 4)*: 937-941.

Yap CC, Ishii K, Aoki Y, Aizaki H, Tani H, Shimizu H, Ueno Y, Miyamura T & Matsuura Y (1997). A hybrid baculovirus-T7 RNA polymerase system for recovery of an infectious virus from cDNA. Virology *231*: 192-200.

Yin P, Cheang M & Coombs KM (1996). The M1 gene is associated with differences in the temperature optimum of the transcriptase activity in reovirus core particles. J.Virol. *70*: 1223-1227.

Zanotto PM, Gibbs MJ, Gould EA & Holmes EC (1996). A reevaluation of the higher taxonomy of viruses based on RNA polymerases. J.Virol. *70*: 6083-6096.

Zeng CQ, Wentz MJ, Cohen J, Estes MK & Ramig RF (1996). Characterization and replicase activity of double-layered and single- layered rotavirus-like particles expressed from baculovirus recombinants. J.Virol. *70*: 2736-2742.

Zheng H, Palese P & Garcia-Sastre A (1996). Nonconserved nucleotides at the 3' and 5' ends of an influenza A virus RNA play an important role in viral RNA replication. Virology *217*: 242-251.

Zhong W, Gutshall LL & Del Vecchio AM (1998). Identification and characterization of an RNA-dependent RNA polymerase activity within the nonstructural protein 5B region of bovine viral diarrhea virus. J.Virol. *72*: 9365-9369.

Zou S & Brown EG (1992). Identification of sequence elements containing signals for replication and encapsidation of the reovirus M1 genome segment. Virology *186*: 377-388.

PUBLICATIONS

Publications emanating from parts of the research presented in this thesis:

Maree, S., Durbach, S., Maree, F.F., Vreede, F. and Huismans, H. (1998). Expression of the major core structural proteins VP3 and VP7 of African horse sickness virus, and production of core-like particles. *Arch. Virol.* [Suppl] **14**: 203-209.

Vreede, F.T., Cloete, M., Napier, G.B., van Dijk, A.A. and Viljoen, G.J. (1998). Sequence-independent amplification and cloning of large dsRNA virus genome segments by poly(dA)-oligonucleotide ligation. *J. Virol. Methods* **72**: 243-247.

Vreede, F.T. and Huismans, H. (1998). Sequence analysis of the RNA polymerase gene of African horse sickness virus. *Arch. Virol.* **143**: 413-419.