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 α32P-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 \( \lambda_3 \) 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 \( \lambda_3 \) was found not to transcribe reovirus-specific dsRNA into ssRNA, nor to replicate plus-strand reovirus RNA into minus-strand RNA. Complexes formed with \( \lambda_1 \) and \( \lambda_2 \) 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 StuI and SmaI 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 BglII sites.

2.1.3 Plasmid constructions: Two oligonucleotides, with sequences 5’ GTTTATTGAGCGATGG 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 pHi8.85, 250 mM KCl, 50 mM (NH₄)₂SO₄; Boehringer Mannheim), 5μl 25mM MgSO₄, 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 (Φ 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.
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 MgCl₂, 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 MgCl₂, 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 1 µg of each template DNA was then mixed with 4 µl 5X transcription buffer (200mM Tris-HCl pH7.9, 50mM NaCl, 30mM MgCl₂, 10mM spermidine; Promega), 2 µl 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 2 µl 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 2 µl transcription reaction mix with 12.5 µl wheat germ extract, 2 µl 1mM amino acid mixture minus methionine, 1 µl 35S-methionine (10mCi/ml), 1.3 µl 1M potassium acetate and 25 units HPRI in 25 µl final volume. Incubation at 28°C for 90 min was followed by SDS-12% PAGE analysis of 10 µl 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 Na₂HPO₄–7H₂O, 0.24g KH₂PO₄ per liter, pH7.4) containing 1mM MgCl₂ (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. 1 ml DMEM per 35mm well was applied and the cells were incubated for 15 to 30 min at 28°C in an atmosphere of 5% CO₂. Thereafter, a mixture of 5 µg CsCl- or Qiagen plasmid kit-purified DNA in 10 µl TE and 10 µl 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 1 ml methionine-free Eagles’ medium supplemented with 60 µCi 35S-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 300 µl 1X PSB (125 mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol) per 35 mm well. 30 µl 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 10 µg of actinomycin D per ml for 30 min at 28°C. The medium was then replaced with 1 ml per well DMEM containing 20 µCi of 3H-uridine per ml and 10 µg actinomycin D per ml, and incubation was continued for 2 h at 28°C in 5% CO2. Total RNA was extracted from the cells as described by the protocols using the Promega RNAgents total RNA isolation system. Cells were lysed in 500 µl denaturing solution per well, with the addition of 1/10 volume 2M NaAc pH 4. This was followed by a phenol/chloroform/isoamyl alcohol extraction and isopropanol precipitation. The washed and dried RNA pellets were resuspended in 20 µl RNase-free water. 5 µl 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 (0.9 M NaCl, 50 mM sodium phosphate, 5 mM EDTA pH 7.7), 5X Denhardt’s solution, 0.1% SDS, 50% formamide). 32P-labelled RNA probe, synthesised by incorporation of 32P-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 NH₂ histidine-tagged VP1 was achieved by PCR modification of the gene in 91.pGEM3z with primers 9p1.1 (5' CGGGATCCATGGTCATCACCGTGCG 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 CaCl₂-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 10⁵ 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 10⁵ 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 ³⁵S-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 10⁸ cells/ml in 0.15M STE-TX buffer (0.01M NaCl, 10mM Tris-HCl pH 7.4, 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.pOR322) 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-x1,y-y1)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, x1 and y1 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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Clone</th>
<th>RNA terminal sequences</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHSV-9 VP1</td>
<td>91(8,4)p</td>
<td>GG-GTTTAT-GTTTAT.</td>
<td>cDNA clone; preferred transcription initiation sequence</td>
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<tr>
<td></td>
<td></td>
<td>..ACTTAC-AATC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>91(2,0)p</td>
<td>GG-GTTTAT..</td>
<td>exact 3' terminus</td>
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<td></td>
<td></td>
<td>..ACTTCAT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>91ΔK(2,0)p</td>
<td>GG-GTTTAT.</td>
<td>Kpn I667 - Kpn I1257 deleted; exact 3' terminus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>..ACTTAC-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>91(2,0-3)p</td>
<td>GG-GTTTAT.</td>
<td>3' terminus deleted</td>
</tr>
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<td></td>
<td></td>
<td>..ACT-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>91(2,0)n</td>
<td>GG-GTAAGT.</td>
<td>(-) sense RNA; exact 3' terminus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>..ATAAAC-</td>
<td></td>
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<tr>
<td>AHSV-9 VP3</td>
<td>93(7-26,5-43)p</td>
<td>GGGATCT-ATGCAA.</td>
<td>start and stop codons underlined; complete open reading frame</td>
</tr>
<tr>
<td></td>
<td></td>
<td>..TACGACCAGC-AGATC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>93(7-43,5-26)n</td>
<td>GGGATCT-GGCTGC.</td>
<td>(-) sense RNA</td>
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<td></td>
<td></td>
<td>..TTGACAT-AGATC</td>
<td></td>
</tr>
<tr>
<td>AHSV-9 NS2</td>
<td>98(2,0)p</td>
<td>GG-GTTTAA.</td>
<td>exact 3' terminus</td>
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<td></td>
<td></td>
<td>..ACATAC-</td>
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<tr>
<td></td>
<td>98(2,0)n</td>
<td>GG-GTATGT.</td>
<td>(-) sense RNA; exact 3' terminus</td>
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<td></td>
<td>..TTAAAC-</td>
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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 λ<sub>HindII</sub> 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 AHHSV-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 AHHSV-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 AHHSV-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.

<table>
<thead>
<tr>
<th>Gene product</th>
<th>VP1</th>
<th>VP1</th>
<th>VP1</th>
<th>VP1mut</th>
<th>VP3</th>
<th>NS2</th>
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<tbody>
<tr>
<td>Replication-competent transcript</td>
<td>X?</td>
<td>✓</td>
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Combination:

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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/10⁶ 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 AHHSV-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 AHHSV-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 λHind 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
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<td>Triton X-100 conc. (%)</td>
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<td>0.5</td>
<td>1</td>
<td>1</td>
<td>-</td>
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<tr>
<td>Fraction</td>
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<td>p</td>
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**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^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 system, 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.
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Potgieter AC. Onderstepoort Veterinary Institute, Private Bag X5, Onderstepoort 0110, South Africa.


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


PUBLICATIONS

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

