Chapter 5

Transfection of cells in culture with *in vitro*-derived rotavirus transcripts

5.0 Introduction

The development of a rotavirus reverse genetics system is complicated due to the critical lack of fundamental knowledge about how rotaviruses replicate and package their genomes (chapter 2; section 2.3). In a recent review, Trask and co-workers suggested that the failure to recover rotavirus by reverse genetics could be due to technical problems (Trask *et al.*, 2012a). However, another review reports that technical problems such as those associated with reagents were excluded based on the fact that the orthoreovirus reverse genetics system could be successfully reproduced (Taniguchi and Komoto, 2012).

Since no rotavirus has been recovered using a complete set of genome transcripts or cDNA, the objective of the experiments presented in this chapter was to study and characterise the general response and viability of cells following the transfection of *in vitro*-derived rotavirus transcripts obtained by two different methods. The first method involved the use of transcripts derived from the consensus genome sequence of the rotavirus DS-1 strain. The use of a rotavirus consensus sequence in an attempt to develop a reverse genetics system has not been performed previously due to the limitations of available methods to determine a consensus sequence. However, in recent years, next-generation sequencing which allows the determination of viral consensus sequences had been developed and has become more affordable and accessible. The strategy for the transcript-transfection approach required the engineering of rotavirus DS-1 consensus cDNAs to ensure that the exact 5'- and 3'-terminal end sequences i.e., 5'-GGC$(A/U)_7$ and UXUGACC-3', where X is G in all genome segments except in genome segments 2 (VP2) and 10 (NSP4) in which it is A (Mlera *et al*., 2011), could be obtained (Figure 5.1) The correct 5'- and 3'-terminal, and UTR sequences are critical since these regions contain *cis*-acting, and possibly *trans*-acting, signals which are vital for rotavirus genome replication, assortment and

packaging (Mitzel *et al*., 2003, Tortorici *et al*., 2006, Patton *et al*., 1996, Li *et al*., 2010).

The second method was similar to that used for the successful recovery of viruses in the bluetongue virus (BTV) and African horsesickness virus (AHSV) systems. The idea was to generate wild type rotavirus SA11 transcripts by *in vitro* transcription using transcriptionally active DLPs (Matsuo *et al*., 2010, Boyce *et al*., 2008, Boyce and Roy, 2007). The wild-type rotavirus SA11 transcripts were transfected into various cell lines under different conditions.

5.1 Materials and methods

5.1.1 Rotavirus DS-1 genome segment design for *in vitro* synthesis of exact mRNAs

The consensus sequence of the rotavirus DS-1 genome was determined with 454® pyrosequencing and presented in chapter 3 (Mlera *et al.*, 2011). Using the consensus sequence, the cDNA of each genome segment was designed to contain the sequence of the T7 polymerase promoter at the 5'-terminal end and either a *BsaI* or a *BsmBI* restriction site at the 3'-terminal end (Figure 5.1). The genome engineering was similar to the approach Boyce and co-workers followed for developing a reverse genetics system for BTV (Boyce *et al.*, 2008). The restriction sites were positioned to obtain exact rotavirus 3'-terminal sequences following restriction enzyme digestion (Figure 5.1). The nucleotide sequences of genome segments 5 (NSP1) and 6 (VP6) contained internal *BsaI* restriction sites within their sequences and were, therefore, designed to contain *BsmBI* restriction sites at the 3'-end.

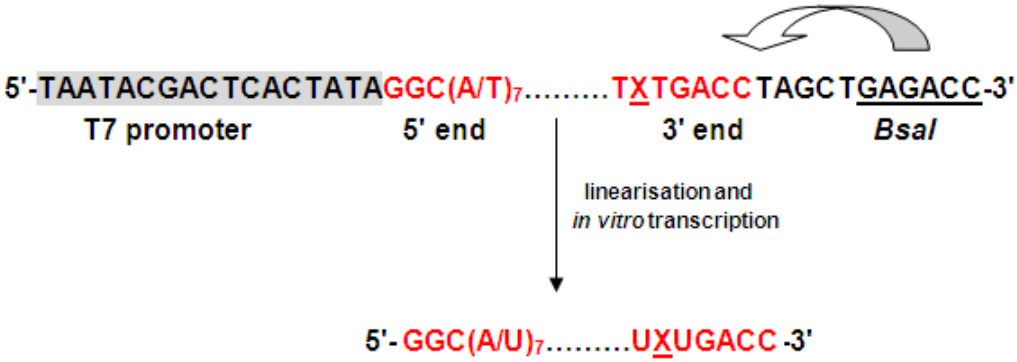

Figure 5.1. Schematic representation of cDNA genome segment engineering to facilitate *in vitro* transcription by T7 polymerase. The T7 promoter sequence is shaded in gray and the 5'- and 3'-terminal sequences of rotavirus DS-1 cDNA genome segment and mRNA are indicated in red. The specific nucleotide sequence recognised by *BsaI* is underlined and the arrow indicates the restriction site which is digested to produce exact 3'-terminal ends.

A *SapI* restriction enzyme site present in genome segment 5 at nucleotide position 1133–1139 was abolished by replacing C with T at nucleotide position 1134, and T with A at nucleotide position 1137. This was performed to create a marker which would be used to differentiate between any rotavirus DS-1 recovered using reverse genetics and the parent rotavirus DS-1 strain. The engineered consensus whole-genome sequence was submitted to GenScript (USA) for commercial genome synthesis.

5.1.2. Reconstitution of the synthetic commercial plasmids containing inserts encoding the rotavirus DS-1 genome

At GenScript, each engineered genome segment was synthesised and cloned into a pUC57 plasmid vector and delivered in a lyophilised form to our laboratory. The pUC57 plasmid contains an ampicillin resistance gene (Figure 5.2). The amount of pUC57 plasmid containing each of the rotavirus DS-1 genome segments was 4 µg. Following the recommendations of GenScript, the vials containing each of the rotavirus DS-1 genome segments in the pUC57 plasmid were centrifuged to avoid loss of material during opening. To prevent contamination, reconstitution was achieved by adding 50 µl previously unopened 10 mM Tris/HCl (pH 8.5) elution buffer (Qiagen). Dissolving of the plasmids was improved by heating at 50 ^{o}C for 15 minutes.

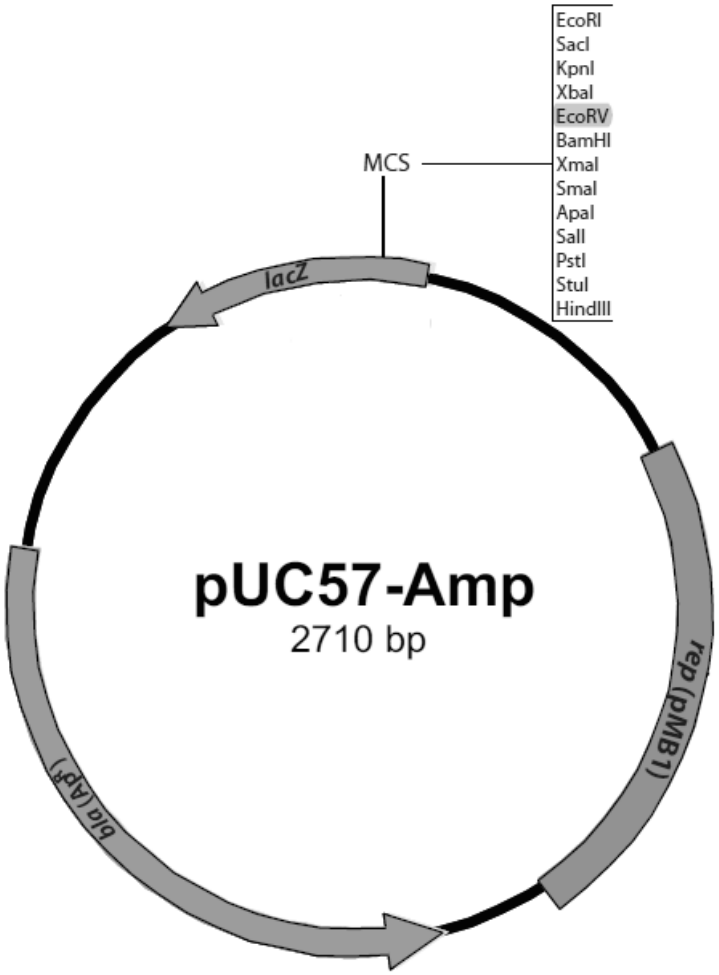

Figure 5.2. Plasmid map of pUC57 (ampicillin resistant) (http://www.genewiz.com).The rotavirus DS-1 genome segments were cloned at the *EcoRV* restriction enzyme digestion site (shaded in gray).

5.1.3 Transformation of ABLE C cells and plasmid amplification

To amplify the plasmids, ABLE C (Stratagene) cells were used. ABLE C cells are capable of reducing copy number 4-fold and were transformed with the pUC57 plasmids containing the rotavirus DS-1 synthetic genome segments. Reduction of the copy number was necessary to reduce potential rotavirus genome segment toxicity in the transformed cells. ABLE C cells were thawed on ice and divided into 20 µl aliquots. To improve the efficiency of transformation, β-mercaptoethanol (Stratagene) was added to a final concentration of 20 mM (Hanahan, 1983). This was followed by incubation on ice for 10 min with swirling every 2 minutes. A total of 0.04 µg of the pUC57 plasmid containing the DS-1 genome segments was added to

an appropriately labelled tube. The cells were incubated on ice for 30 minutes followed by incubation at 37 ^{o}C for 45 seconds and immediately placed on ice for 2 minutes. ABLE C cell-recovery was achieved by adding 200 µl of preheated (42 ^{o}C) super optimal broth with catabolite repression, to each tube followed by incubation at 37 ^{o}C with shaking at 225 rpm (Infors-HT orbital shaker incubator). Transformed ABLE C cells (50µl) were inoculated into 100 ml of terrific broth (TB) containing 100 µg/ml ampicillin (Sigma), 50 µg/ml kanamycin (Sigma) and 10 µg/ml tetracycline (Sigma). Incubation was performed at 37 ^{o}C for 16 hour with shaking at 225 rpm.

5.1.4 Plasmid extraction

To pellet transformed ABLE C cells, centrifugation was performed at 7000 rpm for 15 minutes at 4 ^{o}C (Heraeus). A Qiagen midi plasmid extraction kit was used to extract the various plasmids containing the respective DS-1 rotavirus genome segments following the manufacturer's instructions. The extracted plasmids were dissolved in 100 µl elution buffer (Qiagen). Concentrations of the extracted pUC57 plasmid DNA containing each of the rotavirus DS-1 genome segments were determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific).

5.1.5 Analysis of synthetic whole-genome nucleotide sequences

To verify that the genome segment nucleotide sequences were unchanged following amplification in ABLE C cells, restriction enzyme digestion and Sanger sequencing were performed. Restriction enzyme digestion achieved two goals i.e., (i) linearised the plasmid to verify that the insert was of the correct size; (ii) digested insert and pUC57 to verify general correctness of sequences at specific restriction enzyme site positions. The list of restriction enzymes used is presented in Table 5.1. All enzymes and buffers used were obtained from Fermentas, except *BsaI* which was purchased from New England Biolabs®. The restriction digestions were performed in a 20 µl reaction and each reaction contained 5 U of the appropriate restriction enzyme. *EcoRI* digestions were performed in 1X orange buffer (Fermentas) containing 50 mM Tris-HCl (pH 7.5 at 37 ^{o}C), 10 mM $MgCl_{2,}$ 100 mM NaCl and 0.1 mg/ml BSA. *XhoI*, *HindIII* and the *MluI* with *SspI* restriction digestions were performed in 1X red buffer

(Fermentas) which contained 10 mM Tris HCl (pH 8.5 at 37°C), 10 mM $MgCl_2$, 100 mM KCl and 0.1 mg/ml BSA. The rest of the restriction enzyme digestions were performed in 1X Tango buffer which contained 33 mM Tris-acetate (pH 7.9 at 37°C), 10 mM Mg-acetate, 66 mM K-acetate and 0.1 mg/ml BSA. All restriction enzyme digestions were performed at 37 ^{o}C overnight except *SspI* with *SpeI* restriction enzyme digestions which were performed for 2 hours to prevent star activity. The restriction enzyme digestions were analysed with 1% agarose gel electrophoresis in 1X TAE buffer at 70 volts for 1.5 h. The agarose gel contained 1 mg/ml ethidium bromide to facilitate visualisation of bands using GeneSnap (Syngene) image acquisition software and a ChemiGenius Bio Imaging System (Syngene).

Table 5.1. Restriction enzymes used for restriction digestion analysis of the pUC57 vectors containing the respective 11 different DS-1 rotavirus genome segments.

Genome segment in pUC57 plasmid	Restriction enzyme used for linearization	Multiple restriction enzyme(s)
1 (VP1)	*XhoI*	*HindIII*
2 (VP2)	*SpeI*	*AatII* and *BamHI*
3 (VP3)	*XbaI*	*AatII* and *BamHI*
4 (VP4)	*XbaI*	*SpeI* and *SspI*
5 (NSP1)	*EcoRI*	*SspI*
6 (VP6)	*EcoRI*	*BsaI*
7 (NSP3)	*EcoRI*	*SspI* and *NcoI*
8 (NSP2)	*EcoRI*	*SspI* and *NcoI*
9 (VP7)	*SpeI*	*SspI*
10 (NSP4)	*EcoRI*	*AatII or SspI* and *MluI*
11 (NSP5/6)	*EcoRI*	*SspI*

Sanger sequencing was also performed to verify the correctness of genome segment sequences. Sanger sequencing was performed, at Stellenbosch University's Central Analytical Facilities, DNA Sequencing Unit, using the primers listed in Table 5.2. A set of universal primers (pUC57 U_F and pUC57_U_R) were designed to sequence the regions containing the T7 polymerase promoter sequences and 3'-terminal end sequences. For the large genome segments (genome segments 1–4), internal primers were designed to facilitate sequencing of

the entire genome segments. The universal primer set was sufficient for sequencing genome segments 5–11 since the sequencing products could overlap. The nucleotide sequences obtained with Sanger sequencing were compared to the consensus sequence designed to contain the T7 promoter sequence and restriction enzyme sites using Sequence Viewer v 6.4 (CLC Bio).

Table 5.2. List of primers used to sequence the rotavirus DS-1 genome segment inserts in the pUC57 vector.

Primer ID	Primer sequence	Size (bp)	Nucleotide position	GC content (%)	Tm (^{o}C)
pUC57_U_F[a]	5'-CACGACGTTGTAAAACGACGGC-3'	22	79[b]	54.5	57
pUC57_U _R[a]	5'-AAACAGCTATGACCATGATTACGC-3'	24	77[c]	41.7	54
DS-1_VP1_F1	5'- CCATATTACTTAGTAACATGGGC-3'	23	717-723	39.1	52
DS-1_VP1_R1	5'- GACATCACCATATGATAAGAGC -3'	22	1594-1615	40.9	51
DS-1_VP1_F2	5-GCGAAACATACTAGAGAATATGC-3	23	1547-1569	39.1	52
DS-1_VP1_R2	5'-TATTTGCGCACGTCTCTTCTCG-3'	22	2638-2659	50.0	55
DS-1_VP2_F	5'- AAACGGCTAGCATATGCGACGC-3'	22	700-721	54.5	57
DS-1_VP2_R	5'- GCAATTTTATCTGAGGCACG-3'	20	2172-2191	45.0	50
DS-1_VP3_F	5'- TAGCAAGACTTTCAAATCGCG-3'	21	650-672	42.9	50
DS-1_VP3_R	5'-CTTACCACCTTCAATGCTAGC-3'	21	2000-2020	47.6	52
DS-1_VP4_F	5'-GAAGAGTATGGACATTTCATGGTG-3'	24	600-623	41.7	54
DS-1_VP4_R	5'-TGTTGAAACATCATTCACTGAGTC-3'	24	1892-1915	37.5	52

[a] Universal forward and reverse primers used to bind the pUC57 vector sequences upstream and downstream of the inserts.
[b] Position in pUC57 upstream of insert
[c] Position in pUC57 downstream of insert

5.1.6 *In vitro* transcription of the synthetic rotavirus DS-1 genome

To use the rotavirus DS-1 cDNA as a template for *in vitro* transcription, the pUC57 plasmids (GenScript) were linearised using *BSaI* or *BsmBI* (New England Biolabs®) for genome segments 5 and 6. A total of 15–20 µg of the pUC57 plasmid DNA containing each DS-1 rotavirus genome segment was linearised in a 100 µl reaction with 25 U *BSaI* or *BsmBI*. The *BsaI* restriction enzyme digestion reaction was supplemented with 100 µg/ml bovine serum albumin and the digestion was performed at 37 ^{o}C for 16 hours. The *BsmBI* restriction digestion was performed at 55 ^{o}C for 16 hours. Inactivation of *BsaI* and *BsmBI* was performed by heating for 20 minutes at 65 ^{o}C and 80 ^{o}C, respectively. This was followed by 1% agarose gel electrophoresis in 1X TAE buffer at 70 volts for 2 hours. Excision of the DNA

fragments containing the rotavirus genome segments downstream of the T7 polymerase promoter was performed. The excised fragments were purified using a Qiagen gel extraction kit according to the manufacturer's instructions. The quantity of the linearised cDNA was determined using a NanoDrop® 1000 spectrophotometer.

One microgram of the linearised template was used for *in vitro* transcription with the mMESSAGE mMESSENGER® T7 Ultra kit (Ambion®). The kit contains an anti-reverse cap analogue (ARCA) for combined transcription and capping. Each genome segment was transcribed separately in a 20 µl reaction. The *in vitro* transcription reaction contained 1X T7 NTP/ARCA mix, 5X T7 reaction buffer and 0.5X T7 enzyme mix, 1µg of DNA template and nuclease free water (Ambion®). The *in vitro* transcription reaction was performed at 37 ^{o}C for 2.5–4 hours. The DNA template was destroyed using 2 U of TURBO DNase (Ambion®) and incubation at 37^{o}C for 15 minutes. Following the removal of the DNA template, the rotavirus DS-1 mRNA was cleaned up with a MEGAclear™ RNA purification kit (Ambion®) according to the manufacturer's instructions. Transcripts were eluted in nuclease-free water containing RNasin Plus® (Promega) at 0.5 U/µl. A small aliquot of each transcript was denatured in formaldehyde loading dye (95% formamide, 0.025% SDS, 0.025% bromophenol blue, 0.025% xylene cyanol FF, 0.025% ethidium bromide, 0.5 mM EDTA) (Fermentas) at 70 ^{o}C for 10 minutes followed by denaturing 1% agarose formaldehyde gel electrophoresis in 1X MOPS buffer (0.04 M 3-(N-morpholino)propanesulfonic acid; 0.01 M sodium acetate; 0.001 M EDTA; pH7.0). Following electrophoresis, the transcripts were quantified using a NanoDrop® 1000 spectrophotometer and small aliquots of the transcripts were stored at -80 ^{o}C.

5.1.7 Additional transcript capping reaction

The capping efficiency of mMESSAGE mMACHINE T7 Ultra kit is approximately 80%. To achieve total capping of transcripts, a ScriptCap™ m^{7}G capping system (Epicentre Biotechnologies) was used. The ScriptCap™ capping enzyme was derived from vaccinia virus. The ScriptCap™ capping reaction contained 1–10 µg mRNA, 1X ScriptCap™ capping buffer, 1 mM GTP, 0.01 mM S-adenosyl methionine (SAM), 2 U/µl ScriptGuard™ RNase inhibitor and 10 U ScriptCap™ capping enzyme.

The total reaction volume was 20 µl and the capping reaction was performed at 37 ^{o}C for 0.5–1 hour. Following the additional capping step, mRNA was purified with the MEGAclear^{TM} kit.

5.1.8 Preparation of transcriptionally active rotavirus SA11 DLPs

Rotavirus SA11 virus was propagated in bulk cultures in fifteen 175 cm^2 flasks (Nunc^{TM}). MA104 cells were infected at a multiplicity of infection of 0.3. Propagation of rotavirus SA11 was achieved by incubating rotavirus-infected MA104 cells in DMEM containing 1 µg/ml porcine trypsin IX and supplemented with 1% NEAA and 1% PSA (section 4.1.1). The cultures were harvested when the cytopathic effect (CPE) was at least 70%. Any attached cells were scrapped off and the harvest comprised of the total cells and culture medium. The cellular component of the harvest was separated from the culture medium by centrifugation at 986 x *g* for 5 minutes at 15 ^{o}C to obtain pellet 1. The supernatant was centrifuged at 135 372 x *g*, using a Surespin 630 rotor in a Sorvall ultracentrifuge at 15 ^{o}C, for 1 hour to obtain pellet 2. Pellets 1 and 2 were resuspended in 8 ml of 10 mM Tris/HCl buffered saline (TBS; pH 7.5). A 3.2 ml volume of Vertrel® (DuPont) was added followed by homogenisation of the samples using a glass-teflon Dounce. The homogenates were centrifuged at 438 x *g* at 15 ^{o}C for 5 minutes and pooled. The outer capsid was removed with 10 mM EDTA at 37 ^{o}C for 1 hour (Sandino *et al*., 1986, Patton *et al*., 2002, Estes *et al*., 1979b). The removal of the outer capsid was followed by pelleting the DLPs in a TH641 rotor at 125 000 x *g* for 1.5 hours. The pellet was resuspended in TBS and purified by CsCl (Sigma) gradient centrifugation at 210 000 x *g* for 16 hours at 15 ^{o}C. A pure DLP band was drawn out using a syringe and CsCl removed by dialysis in copious amounts of TBS overnight. An aliquot of the dialysed DLPs was analysed by 10% SDS-PAGE and staining with Coomasie Brilliant Blue R250 (Merck) to verify that DLPs had been obtained (Weber and Osborn, 1969).

5.1.9 *In vitro* transcription of the rotavirus SA11 genome using DLPs

For *in vitro* transcription of the rotavirus SA11 genome, a transcription cocktail was prepared. The cocktail contained 100 mM Tris/HCl (pH 8.0), 50 mM sodium acetate, 10 mM magnesium acetate, 1 mM DTT (Roche), 5 mM rATP (Promega), rCTP,

rGTP, rUTP, at 2.5 mM each, 1 mM s-adenosyl methionine (SAM; Sigma) and 0.4 U/µl RNasin® Plus (Promega). To improve transcription yields in a 100 µl transcription reaction, DLPs were used at approximately 10 µg/ml and 6% PEG_{6000} was added, to increase molecular crowding. The *in vitro* transcription reaction was performed at 40 ^{o}C for 4–6 h (Mason *et al*., 1980, Patton *et al.*, 2002, Cohen *et al*., 1979). Total RNA was extracted from the transcription reaction using TRI-reagent LS (Molecular Research Centre), following a standard RNA extraction protocol. Genomic dsRNA was removed by precipitating ssRNA in 2 M LiCl at 4 ^{o}C for 16 hours followed by centrifugation at 15 000 x *g* for 30 minutes at 4 ^{o}C. The mRNA pellet was washed twice with 75% ethanol followed by dissolving in nuclease-free water (Promega) containing 0.5 U/µl RNasin® Plus. The transcripts were denatured in a formaldehyde loading dye (section 5.1.6) and the integrity of the mRNA was analysed using 1% agarose formaldehyde gel electrophoresis in 1X MOPS buffer at 80 volts for 1.5 hours. The transcript concentration was determined using a NanoDrop™ spectrophotometer (Thermo Scientific) and aliquots were stored at -80 ^{o}C.

5.1.10 Transfection of various cells lines with *in vitro*-derived rotavirus transcripts

BSR, MA104 (kind gifts from Dr. A. C. Potgieter at the Onderstepoort Veterinary Institute) and COS-7 (kindly provided by Dr S. Prince at the Department of Human Biology, University of Cape Town) cell lines were used in attempts to recover viable rotavirus by transfecting *in vitro* derived rotavirus transcripts. COS-7 and MA104 cells were used at a passage between 28 and 40. BSR cells were used at passages between 10 and 30. A day before transfection, cells were seeded in 12- or 24-well plates (Nunc™) to achieve approximately 80–90% confluence the next day. Co-cultures of BSR and MA104 cells were also explored and the cells were seeded at a BSR-cell to MA104-cell ratio of 1: 4. To prevent apoptosis induced by the dsRNA-dependent protein kinase (PKR) 2-aminopurine (Sigma) or imidazolo-oxindole PKR inhibitor C16 (Sigma) was used at 10 nM and 10 µM respectively. PKR induces apoptosis (Balachandran et al., 1998, Gil and Esteban, 2000b, Gil and Esteban, 2000a). Inhibition of PKR was performed for 30 min to 1 h before the transfection was carried out. To prevent oxidative stress induced necrotic cell death, Necrox-2™

(Enzo Life Sciences) was used at 0.12 or 1.2 µM. Necroptotic cell death is a programmed necrotic cell death due to activation of RIP1 (Galluzzi and Kroemer, 2008) and was prevented with Necrox-5™ (Enzo Life Sciences) at 0.12 or 1.2 µM. The inhibition of caspase-mediated cell death was attempted with pre-treating cells using the pan-caspase inhibitor fluoromethylketone, Z-VAD-FMK (Promega) (Yang *et al*., 2004a), at 20 µM for 2 hours.

Lipofectamine® 2000 (Invitrogen) was used for transfecting BSR, COS-7 and MA104 cells, according to the manufacturer's instructions. Various amounts of mRNA and well sizes were investigated with appropriate medium volumes according to the manufacturer's recommendations. A ratio of 1:2.5 (weight: volume) was used to form transcript-Lipofectamine® complexes in the presence of 0.5 U/µl RNasin Plus®. For transfection into various cell lines, all the eleven rotavirus DS-1 transcripts were pooled into equimolar amounts. The complexes were formed at room temperature for 20–30 minutes and applied onto cells covered by a small volume of Opti-MEM® I transfection medium (Invitrogen). An appropriate volume of Opti-MEM I was added and cultures were incubated at 37 ^{o}C and in 5% CO_2. Following transfection for 18–20 hours, a second transfection with all the eleven transcript segments was performed under the same conditions for 4 hours (Matsuo *et al*., 2010). The transfection medium was removed and replaced with DMEM containing 1% penicllin/streptomycin/amphotericin (Lonza), 1% NEAA (Lonza) and 0.25–0.5 µg/ml porcine trypsin IX (Sigma). Variations in trypsin concentrations were based on varying sensitivities of the cells to the protease. As a control for functionality of rotavirus SA11 transcripts, DLPs were also transfected into COS-7 cells under the same conditions followed by passage in MA104 cells. All cultures were observed for the development of CPE and subsequently passaged in MA104 cells to demonstrate if rotavirus had been recovered by reverse genetics.

Single genome segment transcripts of genome segments 4 (VP4), 6 (VP6) and 10 (NSP4) were transfected into COS-7 or BSR cells. Segment 3 (S3; VP3) and segment 6 (S6; VP5) of BTV-1 which has a functional reverse genetics system (Boyce *et al*., 2008, van Gennip *et al*., 2012), were used as controls. The BTV-1 genome segments were kindly provided by Dr Piet van Rijn (Central Veterinary Institute, The Netherlands). The BTV genome segments were under the control of a

T7 promoter inserted in the pUC57 plasmids as described before (Boyce *et al*., 2008). BTV-1 S3 and S6 transcripts were obtained by *in vitro* transcription with mMACHINE mMESSAGE® T7 Ultra kit (Ambion) and purification of transcripts using MEGAclear™ (Ambion) as described in Section 5.1.6. The integrity of single-genome segment transcripts was analysed using 1% agarose formaldehyde gel electrophoresis (Section 5.1.6).

5.1.11 Generation and *in vitro* transcription of defective transcripts

Site-directed mutagenesis of rotavirus DS-1 genome segment 3 (VP3) and BTV-1 S3 (VP3) inserts in the pUC57 plasmids was performed to generate transcriptionally defective capped or uncapped transcripts. The primers used to mutate the genome segment 3 of rotavirus DS-1 were phosphorylated at the 5'-end to facilitate ligation following thermal cycling, and were purchased from Integrated DNA Technologies. The sequences were 5'-TGGTGTAAAC**GCGTA**AGTATTAGC-3' which was engineered to generate a second *MluI* restriction site, and 5'-AGAGGTAAAACACACTACTG-3'. The engineered mutation is bold and underlined. The PCR was performed in a reaction which contained 2 ng cDNA, 2.5 mM of each of the dNTPs (TaKaRa), 1X Phusion® High Fidelity buffer and 1 U Phusion® High Fidelity DNA polymerase (Finnzymes). Following initial denaturation at 98 ^{o}C for 30 seconds, 25 cycles of denaturation at 98 ^{o}C for 10 seconds, annealing at 58 ^{o}C for 30 seconds, chain extension at 72 ^{o}C for 3 minutes, were preformed. For BTV-1 S3, the 5'-phosphorylated mutagenic primers were 5'-ATGAGCAACGTCCGGAACG-3' and 5'-TCTGAGCAGC**TAT**GGCTACGGAA-3' which was engineered to abolish the only *NcoI* restriction site in the plasmid. The engineered mutations are bold and underlined. PCR cycling conditions similar to that used for genome segment 3 of rotavirus DS-1 was used except that annealing was achieved at 67 ^{o}C. The PCR products were analysed using 1% agarose gel electrophoresis in 1X TAE buffer at 70 volts for 1.5 hours. The PCR products were recovered from the agarose gel by excision followed by purification with QIAquick® Gel Extraction kit (Qiagen). To circularise the purified mutant plasmids, ligation was performed using T4 DNA ligase (Fermentas) in a 10 µl reaction that contained 1X ligase buffer (Fermentas) and 15 ng cDNA. Ligation was performed at 22 ^{o}C for 1 hour followed by transformation of

ABLE C cells, plasmid amplification and extraction. The mutated plasmids were analysed by restriction enzyme digestion.

Capped transcripts were transcribed with the mMESSAGE mMACHINE T7 Ultra kit as described in section 5.1.10. Uncapped transcripts were generated using the MEGAscript® T7 kit (Ambion). This was performed for rotavirus DS-1 genome segment 3 (mutant and non-mutant) and 6 as well as BTV1 S3 mutant. The MEGAscript T7 *in vitro* transcription reaction contained 1 µg of cDNA template, 2 µl T7 enzyme mix, 1X reaction buffer, 7.5 mM ATP, 7.5 mM CTP, 7.5 mM GTP, 7.5 mM UTP in a total reaction volume of 20 µl. The transcription reaction was performed at 37 ^{o}C for 3 hours followed by destruction of the DNA template using 2 U of TURBO DNase (Ambion®) and incubation at 37 ^{o}C for 15 minutes. Following the removal of the DNA template, the mRNA was cleaned up, eluted and analysed as described in section 5.1.6. The transcripts were quantified using a NanoDrop® 1000 spectrophotometer and small aliquots of the transcripts were stored at -80 ^{o}C. Transfection of capped or uncapped mutant transcripts was performed with Lipofectamine® 2000 as described in section 5.1.10.

5.1.12 Immunocytochemistry

A day before transfection, COS-7 or BSR cells were seeded into wells of 12-well plates (Nunc™) to achieve 80–90% confluence the next day. The cells were washed twice with PBS followed by incubation with FBS- and antibiotic-free DMEM containing 10 µM imidazolo-oxindole PKR inhibitor C16 (Sigma) for 30 minutes at 37 ^{o}C and 5% CO_2. The cells were transfected with 0.5 µg of rotavirus SA11 mRNA derived from *in vitro* transcription and rotavirus DS-1 genome segment 6 transcripts. An equivalent volume of Opti-MEM® I (Gibco) was used for the mock control. Two transfections were performed, the first for 18 hours and the second transfection for 4 h. Following the second transfection, cells were fixed with 4% formaldehyde (Merck) for 1 hour. The formaldehyde was washed away with PBS three times followed by permeabilisation of the cells with 0.25% Triton X-100 (Merck) at room temperature for 10 minutes. Triton X-100 was washed away with PBS three times followed by blocking of endogenous peroxidase with peroxidase block (Abcam) for 10 minutes at room temperature. Protein blocking was performed with protein block (Abcam)

followed by three washes with PBS. The primary polyclonal antibody raised against rotavirus, Nebraska Calf Diarrhoea Virus strain, NCDV (Abcam) was applied to cover the cells and incubated at room temperature for 2 hours with rocking. The primary antibody was used at a dilution of 1: 250. Five washes in PBS were performed to remove unbound primary antibody followed by the addition of a horse-radish peroxidase-labelled rabbit anti-goat secondary antibody (Dako) for 1 hour at room temperature with rocking. The secondary antibody was used at a 1:500 dilution. Following the removal of unbound secondary antibody, diaminobenzidine substrate was applied for 10 minutes at room temperature followed by three washes with PBS. Immunostained cells were visualised with a Nikon Eclipse TE2000-S microscope and images were captured using the NIS-Elements BR 2.30 software (Nikon).

5.1.13 Western blot analyses

To determine which rotavirus proteins were expressed in BSR cells, 6-well plates (Nunc™ were transfected with 2 µg rotavirus SA11 mRNA (section 5.1.10). Two transfections were performed as described above. Following transfection, cells were washed twice with PBS followed by lysis using 100 µl of 1% NP-40 (Fluka) in a buffer which contained 50 mM Tris and 150 mM NaCl (pH 8.0). The positive controls were lysates of MA104 cells that were infected with rotavirus DS-1 or SA11 at a multiplicity of infection of 1 for 24 hours. The lysates of rotavirus-infected MA104 cells were prepared following infection for 24 hours. The cell lysates were subjected to 10% SDS-PAGE at 25 Amps for 2 hours. Electro-transfer of the proteins onto a nitrocellulose membrane (Whatman®) was achieved at 100 V for 1 hour in a transfer buffer containing 0.025 M Tris, 0.2 M glycine and 20% methanol (pH 8.4). To prevent non-specific antibody binding, the membrane was blocked using 5% skimmed milk (Nestle) in 1X Tris buffered saline with 1% Tween 20™ (TBST; CIS Bio) for 1 hour at 4 ^{o}C with shaking at 140 rpm. Rotavirus proteins were probed by western blot analysis using the antibody (Abcam) raised against the NCDV strain at a 1:1000 dilution. The membrane was incubated with the primary antibody for 16 hours followed by washing four times with TBST. The membrane was incubated with a donkey anti-goat secondary antibody (Abcam) which was diluted to 1:500. Incubation with the secondary antibody was performed at 4 ^{o}C for 1 hour with shaking at 140 rpm. To develop protein bands on the nitrocellulose membrane, a 4-chloro-1-

naphthol peroxidase substrate tablet (Sigma) was dissolved in 10 ml ice-cold methanol followed by adding 2 ml of the dissolved substrate to 10 ml PBS (pH 7.4). Five microlitres of hydrogen peroxide (Sigma) were added to the mixture and the membrane development solution was applied onto the nitrocellulose membrane for 5 minutes or until protein bands had developed sufficiently. To stop further development, the membrane was transferred into deionised water.

5.1.14 Evaluation of cell death pathways

The pathway(s) of cell death induced by the transfection of rotavirus transcripts were investigated using a Cell Death Detection ELISA^{PLUS} kit (Roche) as well as measuring caspase 3/7 activity with the Caspase-Glo 3/7 assay (Promega) in transfected cells. For both approaches COS-7 cells were seeded at a concentration of 2 x 10^4 cells/well, into the wells of a 96-well plate. White-walled plates (Greiner Bio-One) which are designed to prevent well-well crosstalk in luminescent assays were used for the Caspase-Glo 3/7 assay. Transfection was performed in duplicates, with 0.04 µg of either BTV1 S3 mRNA, rotavirus DS-1 genome segment 6 mRNA or rotavirus SA11 DLP-derived mRNA. Two transfections were performed, the first for 20 hours followed by a second transfection for 4 hours, in a transfection medium volume of 50 µl. For the Caspase-Glo® 3/7 assay, an equal volume of Caspase-Glo® 3/7 reagent which contains both a cell lysis reagent and a caspase 3/7 substrate was added and incubated at room temperature for 1.5 hours. The emitted light was measured in relative lights units (RLU) using a Synergy HT multi-mode micro plate reader (BioTek).

For the Cell Death Detection ELISA^{PLUS} assay (Roche), cells in a 96-well plate were centrifuged at 200 x *g* for 10 minutes followed by removal of the supernatant. The cells were lysed with 200 µl lysis buffer (Roche) for 30 minutes at room temperature followed by centrifugation at 200 x *g* for 10 minutes. A volume of 20 µl of lysate cytoplasmic supernatant was transferred into streptavidin coated micro wells (Roche) followed by the addition of 80 µl of an immunoreagent (Roche). The plate was covered with an adhesive film and incubated at room temperature for 2 hours with gentle shaking. The lysate/immunoreagent solution was removed and the wells washed 3X with the Incubation Buffer (Roche) followed by the addition of 100 µl of

2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) solution. The plate was incubated for 10–20 minutes or until sufficient colour development was observed. The absorbance was measured at 405 nm with a Synergy HT multi-mode micro plate reader (BioTek).

5.2 Results

5.2.1 *In vitro* transcription of all 11 synthetic rotavirus DS-1 genome segments

The consensus genome sequence of the rotavirus DS-1 strain was one of the two approaches followed in this study in an attempt to recover rotavirus by reverse genetics. The consensus sequence was used to avoid incorporating minor population variant sequences of the viral population as well as defective transcripts which cannot be expressed (González-López *et al*., 2004, Domingo *et al*., 2012, Domingo *et al*., 2006, Forns *et al*., 1997). To ensure that the correct nucleotide sequence of the cDNA template for each of the 11 rotavirus DS-1 genome segments were used for transcription, the integrity of the pUC57 plasmids containing inserts of the 11 genome segments was tested. This was achieved by restriction enzyme digestion as well as Sanger sequencing of each insert in the pUC57 plasmid as obtained from GenScript. Restriction enzyme digestion of the amplified pUC57 plasmids containing the respective rotavirus DS-1 genome segments revealed digestion patterns that were consistent with *in silico* cloning analyses (Figure 5.3; Appendix 2). However, the restriction enzyme digestion of the pUC57 plasmid containing genome segment 10 (NSP4) with *MluI* and *AatII* initially showed that a very small proportion of the plasmid contained the correct restriction enzyme digestion pattern (indicated by arrow in Figure 5.3B) suggesting a change in the nucleotide sequence. The correct restriction enzyme digestion pattern was obtained following another ABLE C cell transformation (Figure 5.3C). The genome segment 7 (encoding NSP3) transcripts apparently migrated aberrantly slower (Figure 5.4). However, Sanger sequencing (results not shown) confirmed that the sequence of the synthetic rotavirus DS-1 genome, including the T7 promoter and restriction enzyme target sequences, obtained from GenScript following synthesis was identical to that of the consensus sequence determined with pyrosequencing and described in chapter 3.

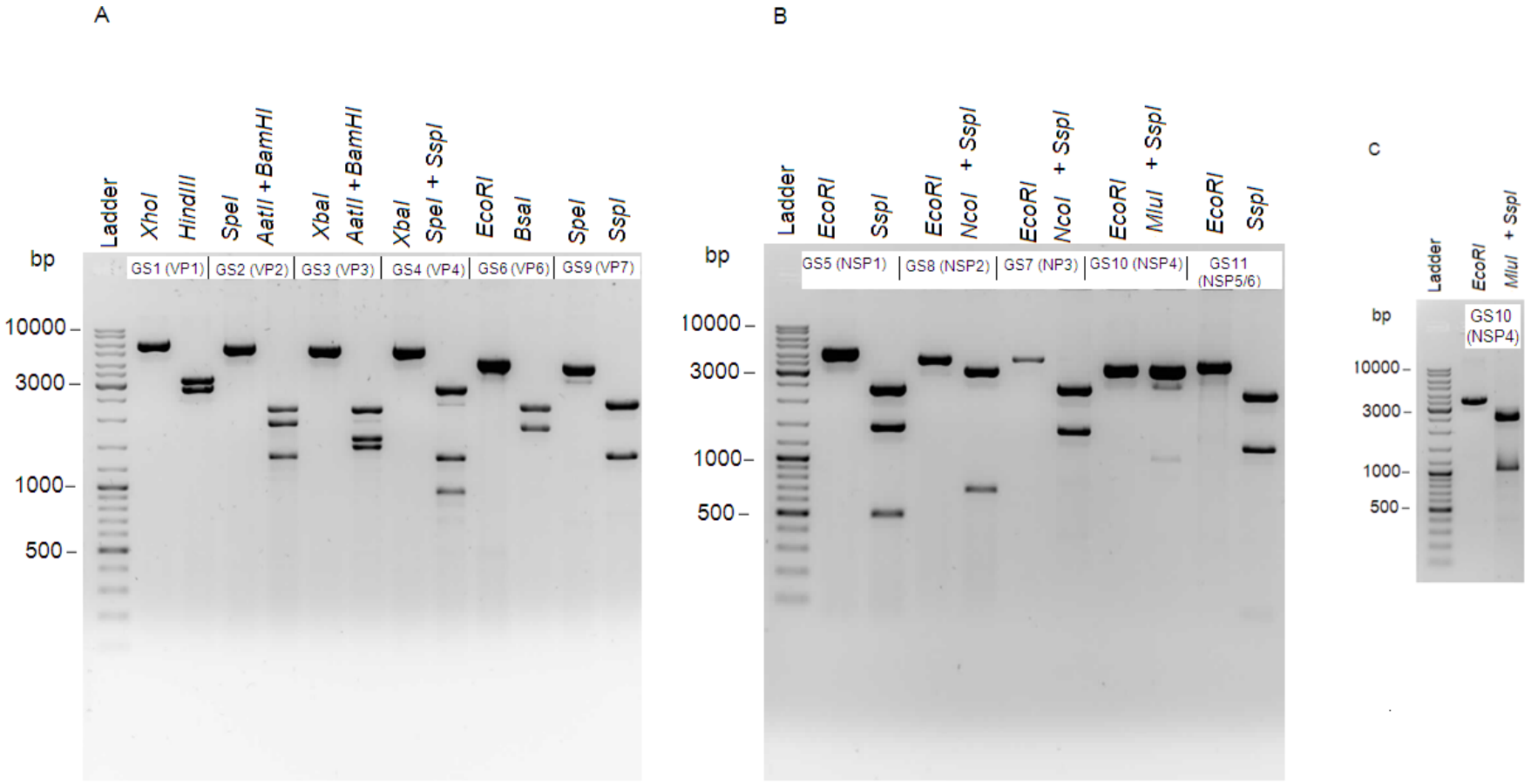

Figure 5.3. Agarose gel (1%) electrophoresis of restriction enzyme digestions of pUC57 plasmids containing synthetic rotavirus DS-1 genome segments. A, Restriction enzyme digestion patterns of pUC57 plasmids containing genome segments 1 (VP1), 2 (VP2), 3(VP3), 4 (VP4), 6 (VP6) and 9 (VP7). **B**, Restriction enzyme digestion of pUC57 plasmids containing genome segments 5 (NSP1), 7 (NSP3), 8 (NSP2), 10 (NSP4) and 11 (NSP5/6). **C**, Restriction enzyme digestion of a second pUC57 plasmid containing genome segment 10 (NSP4) purified from ABLE C cells. The gel shows the expected 1024 bp and 2461 bp fragments obtained from restriction digestion with *MluI* and *SspI* (Appendix 2, Figure 1J). The molecular size marker is an O'GeneRuler DNA Ladder Mix (#SM1173; Fermentas).

The transfection of *in vitro*-derived transcripts of BTV, obtained by transcribing cDNA with T7 polymerase, into appropriate cells results in the recovery of the respective viruses (Boyce *et al*., 2008, van Gennip *et al*., 2012). The transcripts serve two roles as templates for translation into viral proteins and templates for the synthesis of the negative strand which is subsequently packaged as dsRNA(Patton *et al*., 2004). However, it is not established if the same transcripts used for translation are the ones packaged as well. Following the verification that the consensus sequences of the complete set of rotavirus DS-1 cDNA genome segments inserted in pUC57 plasmids were correct, *in vitro* transcription was performed to generate exact rotavirus transcripts. The pUC57 plasmids were digested with either *BsaI* or *BsmBI* to generate linear cDNA templates for the T7 polymerase. *In vitro* transcription using the cDNA templates was successfully achieved for rotavirus DS-1 (Figure 5.4). The amount of transcripts obtained was generally higher for the genome segments encoding structural proteins than genome segments encoding non-structural proteins. The concentration of mRNA obtained from *in vitro* transcription was in the range 180 ng/µl–1650 ng/µl and total yields of 9 µg–82.4 µg.

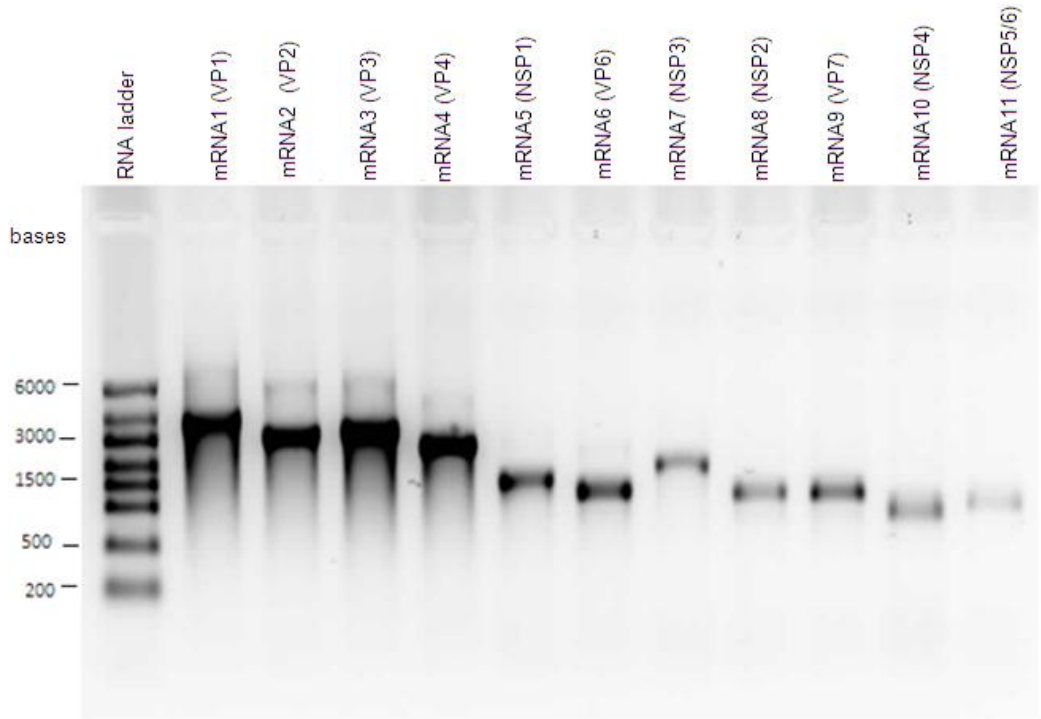

Figure 5.4. Agarose (1%) formaldehyde gel electrophoresis of *in vitro*-derived rotavirus DS-1 transcripts. The rotavirus DS-1 transcripts were individually transcribed from cDNA templates using the mMESSAGE mMACHINE T7 Ultra kit (Ambion®). The transcripts of genome segments 1–11 are identified as mRNA1–11. Lane 1 contains a RiboRuler™ High Range RNA ladder (Fermentas).

5.2.2 Preparation of rotavirus SA11 transcripts using rotavirus SA11 DLPs

Apart from using T7 polymerase to transcribe viral cDNA, BTV and AHSV cores can generate transcripts *in vitro*. The transfection of core particle-derived BTV and AHSV transcripts into appropriate cells also results in the recovery of infectious viruses (Boyce and Roy, 2007, Boyce *et al.*, 2008, Matsuo *et al*., 2010). The transcriptionally active equivalent of BTV and AHSV cores for rotavirus is the double-layered particle (DLP). Therefore, purified SA11 DLPs were required for the *in vitro* transcription of wild-type rotavirus SA11 transcripts. Bulk rotavirus SA11 propagation was performed followed by the removal of the outer capsid proteins (VP4 and VP7). CsCl ultracentrifugation was used to purify the rotavirus DLPs followed by extensive dialysis in 10 mM Tris-buffered saline (pH8.0). Purification of rotavirus SA11 DLPs was successfully achieved as shown by the presence of a single band in a CsCl ultracentrifugation gradient (Figure 5.5A). The DLP protein composition was verified by the identification of the DLP proteins VP1–VP3 and VP6 with 10% SDS-PAGE (Figure 5.5B).

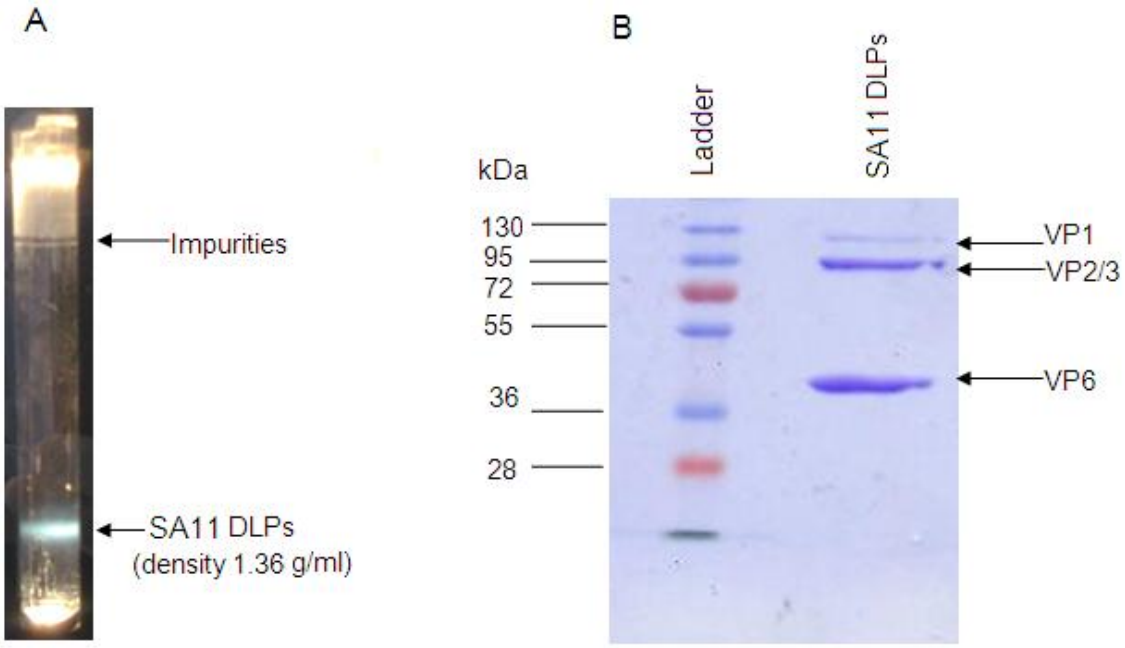

Figure 5.5. Purification of rotavirus SA11 DLPs. A, A band of rotavirus SA11 DLPs (refractive index, 1.37) obtained from CsCl gradient centrifugation**. B,** Verification of DLPs by 10 % SDS-PAGE analysis. The gel was stained with Coomasie Blue R250 and the protein size marker is a PageRuler^{TM} Plus prestained protein ladder (Fermentas).

In vitro transcription, using rotavirus SA11 DLPs, resulted in an average transcription reaction yield of 24 µg. An image of denaturing gel electrophoresis of rotavirus SA11 transcripts is shown in Figure 5.6.

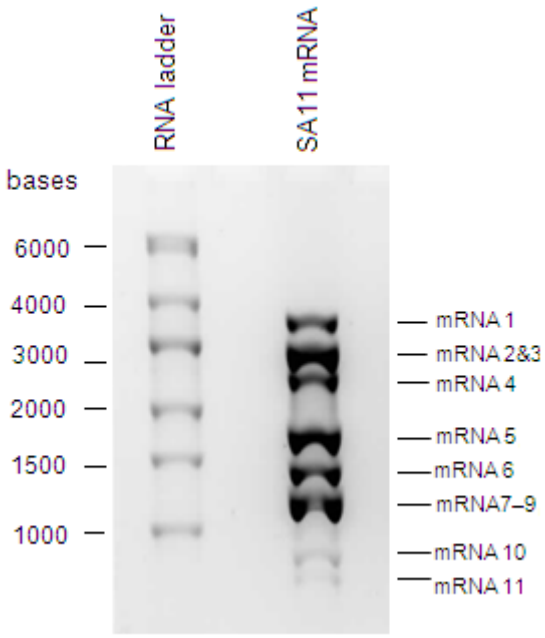

Figure 5.6. Agarose (1%) formaldehyde gel electrophoresis of rotavirus SA11 mRNA transcripts (lane 2) obtained from *in vitro* transcripton using purified rotavirus SA11 DLPs. The mRNAs 1–11 correspond to transcripts from genome segments 1–11. The RNA molecular size marker (lane 1) is a RiboRuler^{TM} High Range RNA ladder (Fermentas).

5.2.3 The effect of transfecting rotavirus DS-1 and SA11 transcripts on cell viability

Many pilot transfections were performed in MA104, BSR, COS-7 and co-cultured MA104 and BSR cells under different conditions. Finally, a two-transfection approach was adopted since it was demonstrated to enhance the efficiency of recovery for the bluetongue virus and African horse-sickness virus (Matsuo *et al*., 2010). For some rotavirus SA11 transcript transfections, the two transfections were performed only two hours apart (Table 5.3) due to the efficient replication in cell culture. MA104 cells, which are the cell-line of choice for rotavirus propagation, could not be transfected efficiently and were not used in any further transfection experiments. COS-7 cells, a monkey kidney epithelial cell line, supports replication of DS-1 and

SA11 rotaviruses (Londrigan *et al*., 2003), can be transfected efficiently and were used for most transfection experiments. BSR cells which were used for the recovery of bluetongue virus (Boyce and Roy, 2007, Boyce *et al*., 2008) and African horse-sickness virus (Matsuo *et al*., 2010) were also transfected.

Following the transfection of both synthetic rotavirus DS-1 and rotavirus SA11 wild-type transcripts, progressive cell death was observed. Tables 5.3 and Table 5.4 present summaries of representative transfection results. Despite the cell death, no viable rotavirus virions were recovered when the cell lysates were blindly passaged up to three times in MA104 cells (Table 5.3; Table 5.4). The additional capping of rotavirus DS-1 transcripts with ScriptCap^{TM} had no impact on cell death and rotavirus recovery. The onset of cell death was slower at lower multiplicity of transfection (MOT) than at high MOT (Table 5.3; Table 5.4). The transfection of rotavirus SA11 DLPs into BSR and COS-7 cells resulted in infectious virus particles which could be propagated in MA104 cells. This was confirmed by the appearance of CPE as well as the extraction of rotavirus dsRNA from the infected MA104 cells (results not shown).

It is well known that viral dsRNA induces autophosphorylation of the dsRNA-dependent protein kinase (PKR) (García *et al*., 2007, Rojas *et al*., 2010). Activated PKR results in modulation of the innate immune response as well as apoptosis and its activity has been inhibited to improve the efficiency of the recovery of bluetongue virus by reverse genetics (Attoui *et al*., 2009, García *et al*., 2007). Despite the inhibition of PKR with 2-aminopurine (2-AP), rapid cell death was observed in COS-7 cells. A more potent and specific PKR inhibitor, imidazolo-oxindole PKR inhibitor C16 (Shimazawa and Hara, 2006, Jammi *et al*., 2003), was used instead of 2-AP. Although cell death was not abrogated when PKR was inhibited by imidazolo-oxindole PKR inhibitor C16, it progressed at a slower rate (Tables 5.3 and 5.4; Figure 5.7). Furthermore, attempts to inhibit cell death with the pan-caspase inhibitor Z-VAD-FMK resulted in aggravated cell death (results not shown).

Table 5.3. Summary of representative transfections performed in various cell lines using synthetic rotavirus DS-1 whole-genome transcripts.

Cells	RNA quantity (µg)	MOT (ng)	Inhibitor	Number of transfections and incubation time	Cell death			CPE (MA104 passage)		
					24h	48h	72h	#1	#2	#3
MA104	600ng/mRNA.	6.1	2-AP	Two: 16h; 4h	No	ND	ND	No	No	No
BSR	Equimolar (2.5 µg)	4.4	None	Two: 4h; 4h	+	++	+++	No	No	ND
MA104/ BSR	Equimolar (2.5 µg)	4.4	None	Two: 4h; 4h	+	++	+++	No	No	ND
COS-7	Equimolar (1.2 µg)	2.1	2-AP	Two: 18h; 4h	+++	++++	ND	No	No	No
COS-7	Equimolar (1.2 µg)	2.1	2-AP	Two: 18h; 4h	+++	++++	ND	No	ND	ND
[†]COS-7	Equimolar (0.17 µg)	0.44	2-AP	Two: 18h; 4h	+++	++++	ND	No	No	No
[†]COS-7	Equimolar (0.25 µg)	0.65	[§]C16	Two: 18h; 4h	+/-	+	++	No	No	ND

MOT, multiplicity of transfection; 2-AP, 2-aminopurine; [§]imidazolo-oxindole PKR inhibitor C16; ND: not performed
[†]Separate capping using the ScriptCap™ m7G capping system (Epicenter) performed after *in vitro* transcription
Cell death grading: +/- CPE at <10%; +CPE at 20-30%; ++ CPE at 50-60%; +++ CPE at 70-80%; ++++ complete CPE

Table 5.4. Summary of transfections performed with *in vitro* DLP-derived rotavirus SA11 transcripts.

Cells	RNA quantity (µg)	MOT (ng)	Inhibitor	Number of transfections and incubation time	Cell death			CPE (MA104 passage)		
					24h	48h	72h	#1	#2	#3
COS-7	0.8	2.1	C16	Two: 4h; 4h	++	+++	++++	No	No	No
COS-7/MA104	0.8	2.1	C16	Two: 4h; 4h	++	+++	++++	No	No	No
COS-7	0.5	1.3	C16	Two: 18h; 4h	+	++	+++	No	No	No
COS-7	1.0	2.6	C16	Two: 4h; 4h	++	+++	++++	No	No	No
COS-7	0.5	1.3	C16	Two: 4h; 4h	+	++	+++	No	No	No
COS-7	0.25	0.65	C16	Two: 4h; 4h	-/+	+	++	No	No	No
COS-7	0.5	1.3	None	Two: 4h; 4h	++++	ND	ND	No	No	ND
COS-7	0.5	1.3	2-AP	Two: 4h; 4h	+++	++++	ND	No	No	ND
COS-7	0.5	1.3	C16	Two: 4h; 4h	+	++	+++	No	No	ND
COS-7	1.0	0.9	2-AP	Two: 4h; 4h	+++	++++	ND	No	No	ND
COS-7	1.0	0.9	C16	Two: 4h; 4h	++	+++	++++	No	No	ND
COS-7	0.5	1.3	C16 +Necrox-2	Two: 18h; 4h	-/+	+	++	No	No	ND
COS-7	0.5	1.3	C16 +Necrox-5	Two: 18h; 4h	-/+	+	++	No	No	ND

MOT, ND as in footnote of Table 5.3
Cell death grading as in footnote of table 5.3.
The light grey shading highlights the degree of cell death for the same amount of transcripts transfected into cells in which PKR was not inhibited, inhibited with 2-AP and oxindolo-imidazole PKR inhibitor C16.

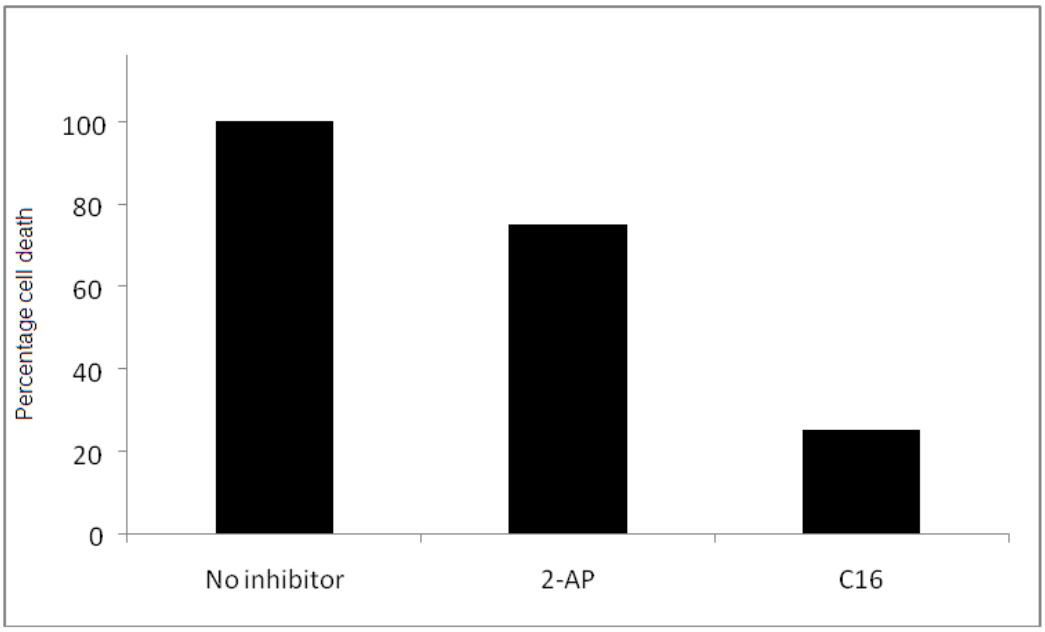

Figure 5.7. Comparison of the effect of 2-aminopurine (2-AP) and imidazolo-oxindole PKR inhibitor (C16) on BSR cell death following transfection with rotavirus transcripts. The comparison was conducted at 24 hours post transfection.

Since the observed cell death was not associated with viral CPE due to lack of viable virus recovery, it was suspected that the rotavirus transcripts could be involved in the induction of cell death. Therefore, the effect of transfecting single genome segment transcripts was analysed. Transfection of *in vitro*-derived mRNA from the cDNA of genome segment 4 (VP4) and genome segment 10 (NSP4) resulted in a similar cell death pattern (Table 5.5). The effect of rotavirus single-genome segment transcript transfection was also compared to synthetic T7 polymerase-derived transcripts of genome segment S3 (VP3) and S6 (VP5) of the bluetongue virus serotype 1 (BTV-1) which also resulted in cell death in both COS-7 cells and BSR cells. The rotavirus DS-1 genome segment 3 was selected for site-directed mutagenesis, following analysis of the open reading frames in all genome segments which showed that translation from the next start codon, should that occur, resulted in significantly shorter product when compared to the original VP3. Furthermore, uncapped mutant transcripts in which the start codon of the open reading frame was removed by site-directed mutagenesis so that translation would not be possible were also used to verify that cell death was induced by the mRNA transcripts and not the expressed proteins. Capped rotavirus DS-1 genome segment 3 (VP3) mRNA, capped mutant genome segment 3 mRNA and uncapped mutated genome segment 3 mRNA also

induced a similar cell death. Uncapped genome segment 6 (VP6) mRNA also induced cell death similar to that observed for genome segments 3 (VP3), 4 (VP4), 6 (VP6) and 10 (NSP4) (Table 5.5).

In COS-7 cells, when PKR was inhibited with imidazolo-oxindole PKR inhibitor C16, BTV-1 transcripts induced cell death similar to rotavirus transcripts. However, the rate of cell death was slower for BTV-1 transcripts (Table 5.5). No difference in the extent of cell death was observed between rotavirus and BTV-1 transcripts following transfection into BSR cells (Table 5.5). The progression of cell death in transfected COS-7 cells with imidazolo-oxindole PKR inhibitor C16 was the same as that observed in BSR cells without PKR inhibition e.g. genome segment 10 (NSP4) transfection in Table 5.5 row 1 and row 5. Cell death was slower in BSR cells transfected with rotavirus transcripts with the inhibition of PKR by imidazolo-oxindole C16 (Table 5.5, row 6). Similarly, a slow cell death pattern was observed when trypsin was excluded from DMEM (result not shown). However, it was observed that there was no cell death induced by rotavirus and BTV-1 transcripts when BSR cells were maintained in DMEM containing 5% FBS following transfection (Table 5.5 rows 7 and 8). However, COS-7 cells maintained in 5% FBS following the removal of the transfection medium continued to die (results not shown).

Table 5.5. Cell death following transfection of single genome segment synthetic transcripts derived from rotavirus and bluetongue virus.

	Cells	DS-1 GS4			DS-1 GS10			DS-1 GS3			DS-1 GS6			BTV1 S3			BTV1 S6		
		24h	48h	72h	24h	48h	72h	24h	48h	72h	24h	48h	72h	24h	48h	72h	24h	48h	72h
1	COS7 +C16	+	++	+++	+	++	+++	ND	ND	ND	+	++	+++	ND	ND	ND	ND	ND	ND
2	COS7 +C16	ND	ND	ND	+	++	+++	+	++	+++	+	++	+++	+/-	+	++	+/-	+	++
3	COS7 +C16	ND	ND	ND	ND	ND	ND	+	++	+++	ND	ND	ND	+/-	+	++	ND	ND	ND
4	COS7 +C16	ND	ND	ND	ND	ND	ND	+	++	+++	ND	ND	ND	+/-	+	++	ND	ND	ND
5	BSR	ND	ND	ND	+	++	+++	+	++	+++	ND	ND	ND	+	++	+++	+	++	+++
6	BSR + C16	ND	ND	ND	+/-	+/++	+++	+/-	+/++	+++	ND	ND	ND	+/-	+/++	+++	+ /-	+/++	+++
7	BSR	ND	ND	ND	-	-	+/-	-	-	+/-	ND	ND	ND	-	-	+/-	-	-	+/-
8	BSR + C16	ND	ND	ND	-	-	+/-	-	-	+/-	ND	ND	ND	-	-	+/-	-	-	+/-

0.5µg of each transcript was used for transfection per well of a 12-well plate
C16, imidazolo-oxindole
GS, genome segment
ND, experiments not performed
BTV1 S3, bluetongue virus serotype 1, segment 3 encoding innermost capsid protein (VP3)
BTV1 S6, bluetongue virus serotype 1, segment 6 encoding inner layer protein of outer capsid (VP5)
Observed cell death grading as indicated in footnote of Table 1
Purple shading indicates transfection of uncapped genome segment 6 mRNA (VP6)
Blue shading indicates transfection performed with capped mutant transcripts
Grey shading indicates transfections performed with uncapped transcripts that were crippled to prevent translation
Green shading indicates replacement of transfection medium with DMEM containing 5% FBS, 1% NEAA, 1% antibiotics (penicillin/streptomycin/amphotericin)

The observation of cell death and that imidazolo-oxindole seemed to slow the cell death suggested that the cell mortality was regulated in response to the transfected transcripts. Some of the regulated cell death pathways include apoptosis and necroptosis (Trump *et al*., 1997, Galluzzi and Kroemer, 2008). Therefore, it was decided to determine if cell death was a result of necrosis or apoptosis. A cell death ELISA which determines levels of histones and DNA and a caspase 3/7 assay were used in this determination. Caspases 3 and 7 are executors of apoptosis (Lakhani *et al.*, 2006). To rule out the possibility of necroptotic cell death, necrosis/necroptosis inhibitors NecroX^{TM}-2 and Necrox^{TM}-5 were used but failed to prevent cell death (Table 5.4). Further characterisation of the cell death with the cell death ELISA suggested that the apoptotic pathways were involved (Figure 5.8A). Recalculation of caspase 3/7 activity, by subtracting the value obtained for the mock control, showed high caspase 3/7 activity (Figure 5.8B).

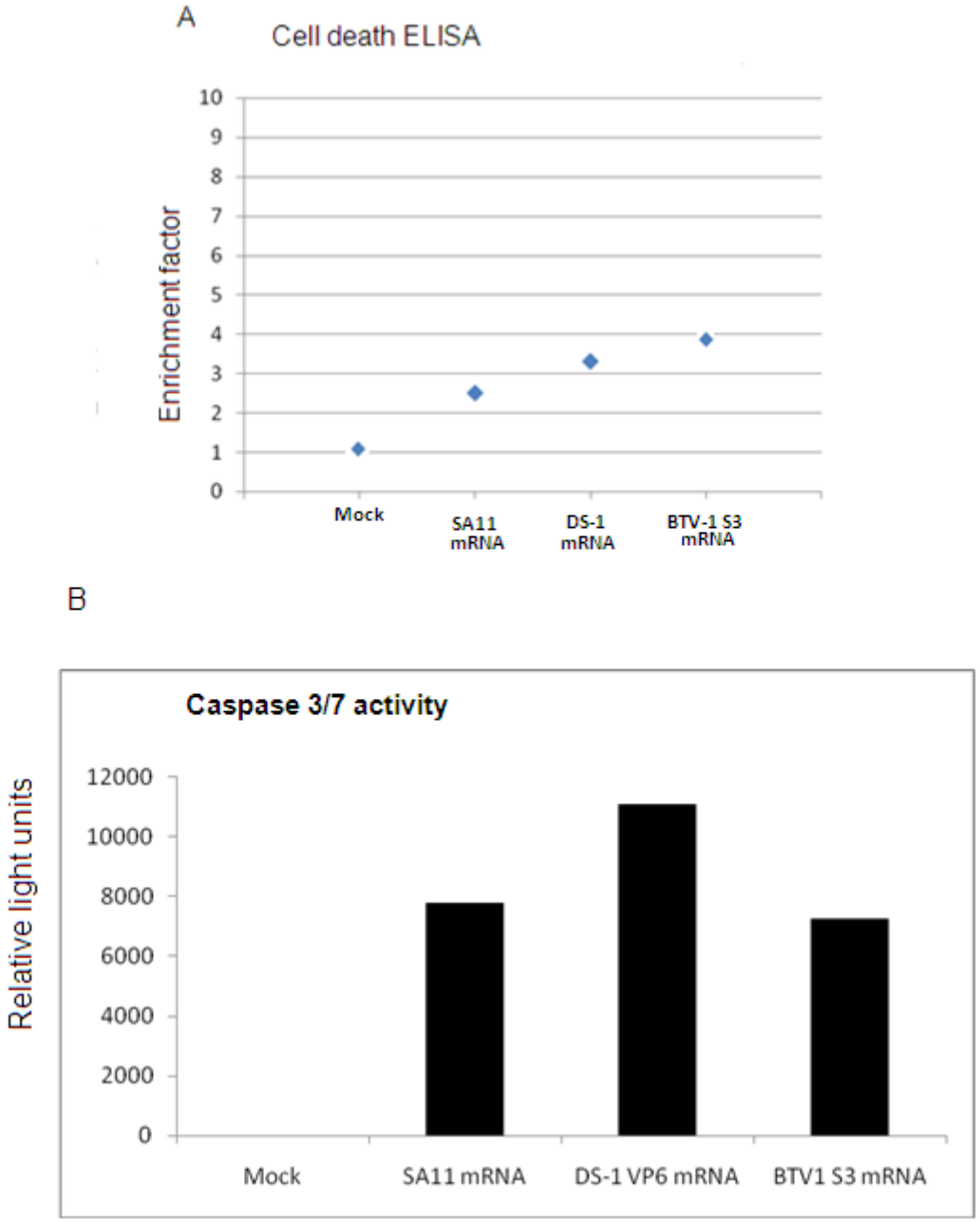

Figure 5.8. Evaluation of cell death mechanisms in transfected COS-7 cells. **A,** Enrichment of apoptosis markers (histones and DNA). **B**, Caspase 3/7 activity in COS-7 cells transfected with various viral mRNAs. The final relative light units indicated were obtained after subtracting the mock value.

5.2.4 Detection of the translation of *in vitro*-derived rotavirus transcripts after transfection into BSR and COS-7 cells

For a transcript-based reverse genetics system to function, of the requirements is the translation of transcripts into proteins. For instance, the RNA-dependent RNA polymerase VP1 and methyltransferase VP3 will function in the synthesis and

capping of nascent transcripts and genome replication (Estes and Cohen, 1989, Patton, 2001). The other structural proteins such as NSP1 function in modulating the innate immune system by down regulating interferon regulatory factors (Barro and Patton, 2005, Barro and Patton, 2007). To determine if the rotavirus transcripts were translated following transfection, rotavirus protein expression was determined using a polyclonal antibody generated against the bovine Nebraska calf diarrhoea disease virus (NCDV) strain. High protein expression levels were observed in BSR cells (Figure 5.9A). In COS-7 cells, rotavirus SA11 transcripts were also translated, but the level of expression appeared lower when compared to that observed in BSR cells (Figure 5.7A). Similarly, the expression of rotavirus DS-1 genome segment 6 mRNA appeared high in BSR cells. In COS-7 cells, the expression of rotavirus DS-1 genome segment 6 mRNA was not detected by immunocytochemistry. Western blot analysis of cell lysates obtained following infection of MA104 cells with rotavirus SA11 showed that the NCDV antibody detects VP6 and VP7 of the rotavirus SA11 strain. For the rotavirus DS-1 strain, only VP6 could be detected (Figures 5.9A; 5.9B). However, western blot analysis of BSR cell lysates transfected with rotavirus DS-1 or SA11 mRNA failed to show the specific rotavirus proteins which were detected by immunocytochemistry (Figure 5.9B).

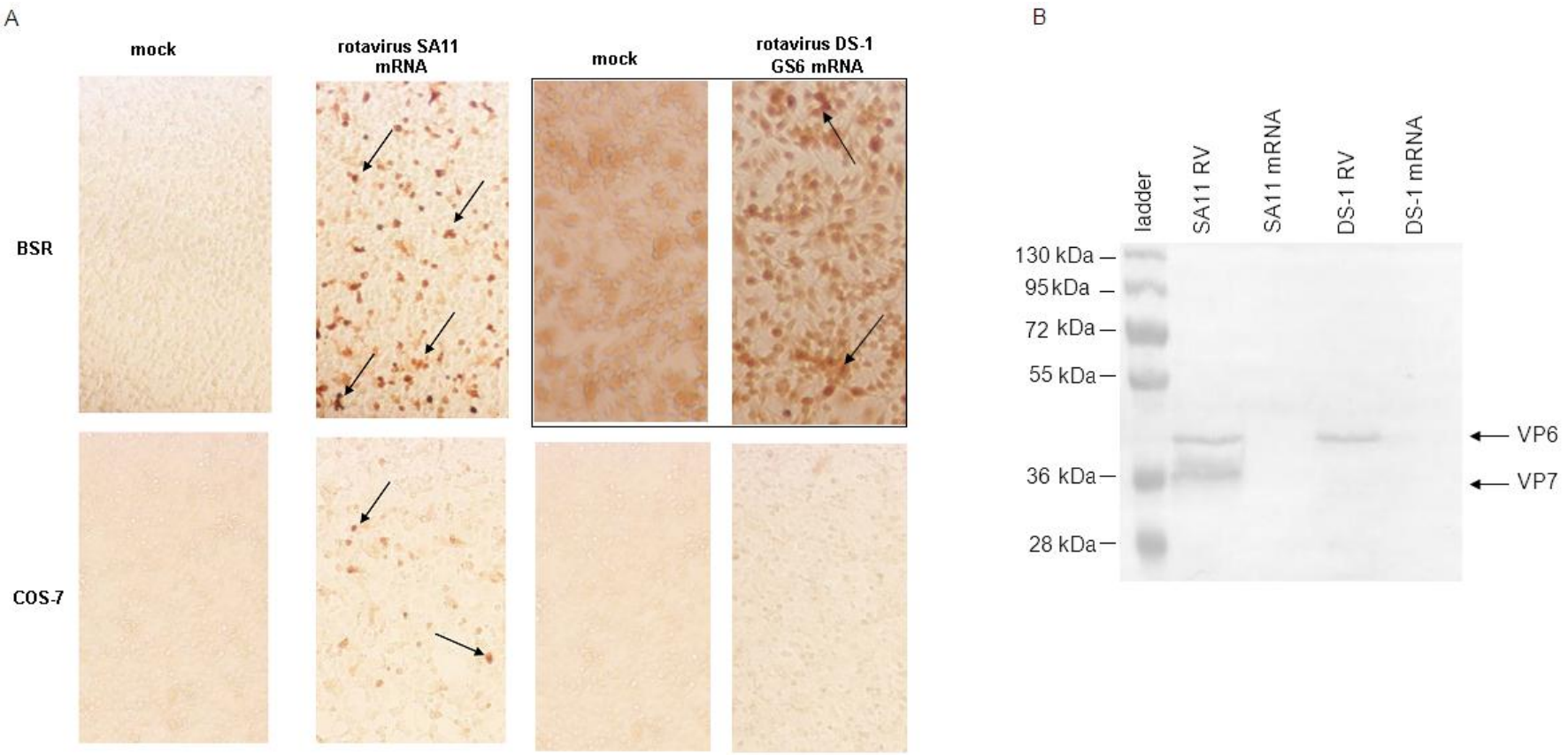

Figure 5.9. Immunological detection of rotavirus protein expression. A, Cells immunostained with anti-rotavirus (NCDV strain) antibody showing the detection of rotavirus proteins in BSR and COS-7 cells following the transfection of *in vitro*-derived rotavirus transcripts. The images of the cell preparations were viewed at a magnification of x100 except for BSR cells transfected rotavirus DS-1 GS6 mRNA which were viewed at X400 (boxed). **B**, Western blot analysis following the infection of MA104 cells with rotavirus DS-1 or SA11, and BSR cells transfected with rotavirus DS-1 mRNA or SA11 mRNA. Lanes with MA104 cells which were infected with are labelled SA11 RV and DS-1 RV, respectively. The protein size marker in lane is a PageRuler^{TM} Plus prestained protein ladder which was transfered onto the membrane together with the viral proteins (Fermentas).

5.3 Discussion

Recent reports have described the development of reverse genetics systems for AHSV and BTV using *in vitro* derived transcripts from core particles (Boyce and Roy, 2007, Boyce *et al*., 2008, Matsuo *et al*., 2010). This study sought to determine if *in vitro*-derived rotavirus transcripts obtained from the consensus sequence cDNA of rotavirus DS-1 and rotavirus SA11 DLPs could be used to recover viable rotavirus. A seemingly characteristic cell death pattern was observed in response to both types of transcripts. However, rotavirus could not be recovered.

The presence of viral dsRNA of at least 30 bp in cells activates the dsRNA-dependent kinase (PKR) component of the innate immune system which subsequently phosphorylates the eukaryotic initiation factor 2α (eIF2α) (Dever *et al*., 2007, Lemaire *et al*., 2008). PKR activation can also occur due to hairpins and loops in ssRNA as well as 5'-triphosphates (Dever *et al*., 2007, Nallagatla *et al*., 2007). The phosphorylation of eIF2α suppresses translation which, in turn, induces apoptosis (Polunovsky *et al*., 1994, Saelens *et al*., 2001). Therefore, a PKR inhibitor such as 2-aminopurine (2-AP) (Marcus and Sekellick, 1988) was used to circumvent cell death following the transfection of transcripts. However, the cell death observed following transfection of rotavirus mRNA in this study, despite PKR inhibition by 2-AP, suggests that this inhibitor did not effectively inhibit PKR in the transfected cells (Figure 5.7). The effectiveness of 2-AP was found to be selective and reversible over incubation time (Marcus and Sekellick, 1988) and this may be the reason for the lack of cell death inhibition when 2-AP was used to inhibit PKR. Cell death was significantly more inhibited using imidazolo-oxindole PKR inhibitor C16, but could not be suppressed completely (Tables 5.3 and 5.4; Figure 5.7). Although imidazolo-oxindole PKR inhibitor C16 slowed cell death, its failure to completely prevent cell death suggests that other PKR-independent cell death mechanisms may also be involved. The contribution of the PKR system towards cell death was demonstrated by the rapid cell death which was observed when cells were transfected without a PKR inhibitor, in comparison to the reduction in the observed cell death when PKR was inhibited with imidazolo-oxindole PKR inhibitor C16. Previously, it was shown that recovery of BTV is enhanced when the PKR system is inhibited (Attoui *et al*., 2009). Since Z-VAD-FMK prevents caspase-dependent cell death, its failure to

prevent apoptosis following the transfection of rotavirus transcripts probably induced necrotic cell death as was reported previously for keratocytes (Kim *et al*., 2000). Taken together, cell death induction by the stimulation of the innate immune response following the transfection of rotavirus transcripts may be of such a nature that the inhibition of a certain cell death pathway could force an alternative pathway towards cell death (Kim *et al*., 2000, Pataer *et al*., 2009).

The transfection of transcripts of single genome segment was performed to demonstrate that the cell death observed was not restricted to a specific genome segment such as genome segment 10 (Table 5.5). The translation product of genome segment 10 (NSP4) is known to be an enterotoxin (Horie *et al*., 1999, Tian *et al*., 1996, Ball *et al*., 1996). Several rotavirus single RNA segment transcripts, including NSP4-encoding transcripts, induced a similar pattern of cell death. The slower progression of cell death, combined with better expression of rotavirus transcripts in BSR cells suggests that the RIG-I deficiency conferred some tolerance of rotavirus transcripts in these cells. The use of trypsin in medium to enhance infectivity of any recovered rotavirus following transfection could also contribute towards cell death. The modification of VP4 trypsin cleavage sites to a furin-sensitive form by rotavirus single genome segment reverse genetics did not improve rotavirus replication efficiency (Komoto *et al.*, 2011). Therefore, it was concluded that the cell death observed was not due to a toxic translation product but a direct response to the rotavirus transcripts. Therefore, the cell death could be the end-result of an antiviral state induced by the viral transcripts. Two possible factors for the cell death were identified. Firstly, RIG-I deficient cells provided more efficient transcript translation which will be required for virus recovery by reverse genetics. Secondly, the absence of cell death when the transfection medium was replaced with DMEM containing FBS (Table 5.5) suggests that FBS probably conferred some protection from cell death.

Recently, Trask and co-workers suggested that the failure to recover rotavirus by reverse genetics approaches similar to those used for the other *Reoviridae* family members could be a technical problem rather than a biological one (Trask *et al*., 2012a). In contrast, Taniguchi and Komoto also recently reported that they were able to exclude problems with reagents or technique in their laboratory (Taniguchi and

Komoto, 2012). For a plasmid-based rotavirus reverse genetics approach, they suggested that there could be an efficiency-related problem associated with the 11th genome segment which is not present in the dsRNA viruses with a genome consisting of 10 genome segments (Taniguchi and Komoto, 2012). Based on the belief that some modifications may be necessary, investigations utilising various cell lines (COS-7, 293T, MA104, CV-1, HT-29, MDCK, Vero, BHK, CHO and L929) and cells constitutively expressing T7 polymerase were unsuccessfully carried out (Taniguchi and Komoto, 2012). The recent *in vitro*, cell-free, reconstitution of the bluetongue virus showed that ssRNA was essential for driving the particle assembly reaction (Lourenco and Roy, 2011). It is not known whether this would be true for rotavirus or that a specific protein may be essential for coordinating mRNA into the next step in the replication cycle and this requires to be investigated.

The failure to recover rotavirus from the transfection of synthetic rotavirus DS-1 or wild-type rotavirus SA11 transcripts in this study could be attributable to several factors in the replication cycle or in the transfected cells. For instance, the formation of viroplasms and genomic dsRNA synthesis for packaging may have failed. In addition to determining that the transcripts are translated, it will be important to demonstrate the formation of viroplasms and genomic dsRNA synthesis. The formation of the viroplasms could be achieved by using NSP2 or NSP5-specific antibodies (Fabbretti *et al.*, 1999, Contin *et al*., 2010, Carreno-Torres *et al*., 2010, Cheung *et al*., 2010). Genomic dsRNA synthesis could be detected with the J2 antibody which is specific for dsRNA (Rojas *et al*., 2010, Weber *et al*., 2006). However, dsRNA detection needs a highly sensitive technique (such as immunofluorescence) since it is likely that genomic dsRNA, if formed, could be in extremely low amounts. Therefore, it is required to verify the expression of all 11 rotavirus proteins for which antibodies need to be generated. The cellular factors probably include a potent innate immune response against the rotavirus transcripts. This is based on the different transcript-tolerance between BSR and COS-7 cells as well as slowed cell death following the inhibition of PKR with imidazolo-oxindole PKR inhibitor C16.

Rotavirus protein expression was detected in BSR and COS-7 cells (Figure 5.9A). This indicates that the rotavirus transcripts were translated. However, no virus

recovery could be demonstrated by using BSR and COS-7 cell lysates to infect MA104 cells. Visual inspection suggested that the expression of the rotavirus transcripts seems to have been higher in BSR cells than in COS-7 cells. This difference could be attributed to differences in the innate immune response of BSR and COS-7 cells. BSR cells are RIG-I deficient (Habjan *et al*., 2008). However, the back-transfection of BSR cell lysates from transfected BSR cells did not result in recovery of rotavirus particles that could infect MA104 cells. The back-transfection was performed based on the idea that any rotavirus recovered in BSR cells could replicate more efficiently in the BSR cells in which the virus would have been recovered from. Since we have not been able to propagate rotavirus in BSR cells in our laboratory, possibly due to lack of specific receptors, it was difficult to test if there were any rescued rotavirus particles that preferred BSR cell replication only.

Western blot analysis showed that the anti-rotavirus (NCDV strain) antibody detects VP6 of both rotaviruses DS-1 and SA11 as well as VP7 of rotavirus SA11 (Figure 5.9B). However, it could not be determined if each of the 11 rotavirus SA11 transcripts were actually translated into proteins. The failure to detect any rotavirus proteins in lysates of transcript-transfected BSR cells by western blot could have been due to very low amounts of protein expressed in the 6-well plate wells. VP1 and VP6 of rotavirus SA11 have the same genotype as that of the NCDV strain (Matthijnssens *et al*., 2008a). Based on the reactivity of the anti-NCDV antibody with VP1 and VP6 of purified rotavirus SA11 DLPs which was shown by western blot and the detection of the expression of rotavirus DS-1 VP6 by immunostaining, it was concluded that there was definite expression of these proteins. Rotavirus strain-specific antibody panels will be needed to determine the expression the other proteins.

In summary, the transcripts of the consensus sequence of rotavirus DS-1 and wild-type rotavirus SA11 transcripts were transfected into BSR, COS-7 and MA104 cells. MA104 cells were abandoned due to poor transfection. The transfected rotavirus transcripts were translated in BSR and COS-7 cells. The seemingly higher expression in BSR cells than in COS-7 cells suggests that the defective RIG-I system in BSR cells provides a transcript-translation advantage and potentially virus recovery as well. This suggests that the rotavirus transcripts may be sensed via the

RIG-I pathway of the innate immune system. The transfection of rotavirus transcripts induces progressive cell death which cannot be prevented with PKR inhibition. The PKR system is only one part of the innate immune system which is involved in sensing pathogen-associated molecular patterns (PAMPs) such as viral nucleic acids. The binding of PAMP receptors to their respective ligands leads, via a cascade of secondary and tertiary factors, to the production of antiviral cytokines such as interferon (Perry *et al*., 2005). The other ligands involved in sensing viral nucleic acids include RIG-I, melanoma differentiation-associated gene 5 (MDA5) and laboratory of physiology and genetics-2 (LPG-2) (Gitlin *et al*., 2010, Yoneyama *et al*., 2005). Taken together, the possible involvement of the RIG-I and PKR systems indicates that a broader innate immune response is pivotal in the rotavirus transcript-induced cell death which appears to be apoptotic rather than necrotic. Cell death is probably the end result of an innate immune response-mediated antiviral state induced in response to the transcripts. The induction of the antiviral state could be very early and potentially prevents rotavirus particle formation. Therefore, the innate immune response to rotavirus transcripts required to be investigated. Chapter 6 presents the findings of this investigation.