CHAPTER 5: INVESTIGATION INTO THE ROLE OF PLASMID DERIVED ROTAVIRUS PROTEINS IN THE DEVELOPMENT OF A ROTAVIRUS TRANSCRIPT BASED REVERSE GENETICS SYSTEM

5

5.1 Introduction

A transcript-based approach to a reverse genetics system for members of the *Reoviridae* family involves the generation of infectious virus particles in cultured cells by using transcriptively active plus-sense ssRNA. Reovirus was the first orthoreovirus to be recovered by transfecting dsRNA and/or ssRNA of the ST3 strain together with co-expressed reovirus proteins (Roner et al., 1990). Infectious virions of two other members of the *Reoviridae* family, bluetongue virus and African horsesickness virus (both *Orbivirus* genus), were also successfully recovered from the complete set of viral mRNAs obtained from transcriptionally active viral cores (Boyce and Roy, 2007, Matsuo et al., 2010). Later on, infectious viruses could be recovered from synthetic RNA transcripts for both bluetongue virus and African horsesickness virus (Boyce et al., 2008, Kaname et al., 2013). The success of these systems was anticipated to be easily emulated onto other members of the *Reoviridae* family, like rotavirus. This was not the case and no transcript-based reverse genetics system currently exists for rotavirus. In a recent comprehensive study by Richards and colleagues, it was concluded that rotavirus transcripts are neither translated nor infectious (Richards et al., 2013).

Similarly, previous studies conducted in our laboratory did not result in the generation of viable rotavirus from a transcript-only based reverse genetics approach (Mlera, 2013). Mlera utilised synthetic transcripts derived from the consensus genome sequence of the rotavirus DS-1 strain as well as an *in vitro* double-layered particle (DLP) derived rotavirus SA11 transcripts in an attempt to establish a transcript-based reverse genetics system. The success of a transcript-based reverse genetics system will most likely depend on three main requirements: the effective translation of transcripts into all 12 rotavirus proteins; the generation of plus-sense ssRNA templates of all 11 genome segments with exact 5’ and 3’ terminal ends for dsRNA synthesis and the efficacious packaging of the dsRNA genome segments to produce a viable rotavirus particle.

In this chapter, the potential of specific plasmid expressed rotavirus proteins to aid in development of a rotavirus transcript-based reverse genetics system will be investigated. Rotavirus SA11 transcripts will be generated through *in vitro* transcription using transcriptionally active DLPs followed by the transfection into several mammalian cell lines (MA104, BSR, COS-7 and HEK 293H). These cultured cell lines were chosen because they all support the propagation of rotavirus SA11 and Wa strains (***Chapter 4***). Two plasmid sets, used for expression of rotavirus proteins, were designed using the consensus sequences of the WaCS strain (RVA/Human-tc/USA/Wa/1974/G1P[8]) (Wentzel et al., 2013) (***Chapter 3***) and SA11 strain (RVA/Simian-tc/ZAF/SA11/1958/G3P[2]) (Mlera, 2013). The rotavirus Wa and SA11 strains were selected for plasmid-based expression systems due to the fact that they propagate well in MA104 cells. Furthermore, the consensus sequences are known for these strains. The Wa constructs comprises of 11 plasmids, each coding for a single rotavirus gene, while in the SA11 constructs, all 11 genome segment inserts were combined into 4 plasmids (Kobayashi et al., 2010). The Wa plasmids can be individually expressed to evaluate the effect of each protein on the transfection of transcripts and/or cell viability. The SA11 plasmids can co-express various proteins which can be used to identify their effect on the transfection of transcripts and influence on cell death. In addition, a plasmid system, codon-optimised for expression in MA104 cells, was designed in an attempt to produce a rotavirus SA11 replication complex scaffold. The hypothesis is that when rotavirus transcripts are transfected in the presence of pre-existing core replication complex (VP1, VP2, VP3 and VP6), transcripts may be recruited into the existing cores (McDonald and Patton, 2011), making virus recovery easier.

5.2 Materials and Methods

5.2.1 Description of Wa and SA11-based expression plasmids

Inserts for the rotavirus Wa and SA11 plasmids were designed to include all 11, full length, genome segments. These plasmids were originally designed for the development of a plasmid-only based reverse genetic system. Each insert was engineered to contain a T7 promoter region, HDV ribozyme region and a T7 termination region. However, due to a design flaw, the T7 promoter sequence of these plasmid systems possessed three additional base pairs on the 3'end (instead of 5'-`TAATACGACTCACTATA-3'` the T7 expression

promoter sequence of 5'-TAATACGACTCACTATAGGG-3' was used containing three additional guanines on the 3'end) (***Figure 5.1***). The mRNA derived from these constructs will have 3 additional guanines before the 6 conserved 5'-terminal residues (5'-GGGGCTTTT…-3') and will most probably not be packaged. This alteration influenced the 5' end of the rotavirus genome segment insert and would likely disrupt replication and packaging of plus-sense ssRNAs. Consequently, the rotavirus Wa and SA11 constructs can only function as expression plasmids.

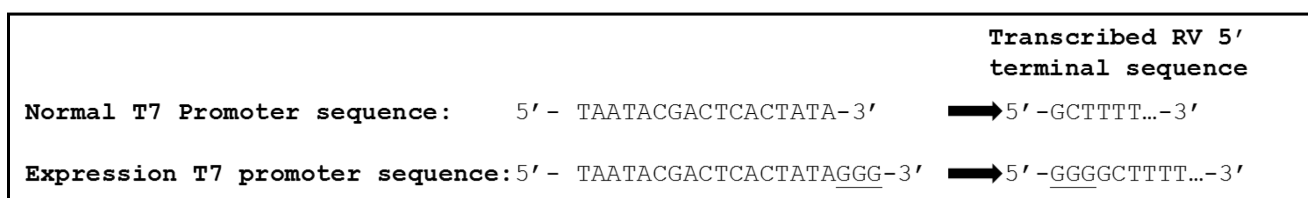

Figure 5.1: Comparison between a normal T7 promoter sequence and the expression T7 promoter sequence used in the rotavirus Wa and SA11 constructs and the resulting rotavirus 5'-terminal sequences.

Every insert is flanked by a unique blunt end restriction enzyme site (***Figure 5.2***. For the rotavirus Wa system, the 11 genome segment cassettes were synthesised and cloned into a pUC57 (GenScript) plasmid vector by GenScript (Piscataway, USA). Each plasmid contained one rotavirus Wa genome segment.

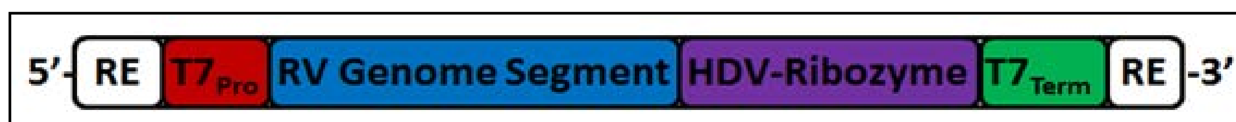

Figure 5.2: An overview of the general rotavirus insert design and plasmid composition*. Each rotavirus genome segments was placed under control of a T7 promoter region, followed by a HDV ribozyme region and a T7 termination region.*

The SA11 cassette design was similar to that of the Wa plasmid constructs, but the eleven genome segment cassettes were grouped into 4 plasmids (Alpha – Delta) (***Figure 5.3).***

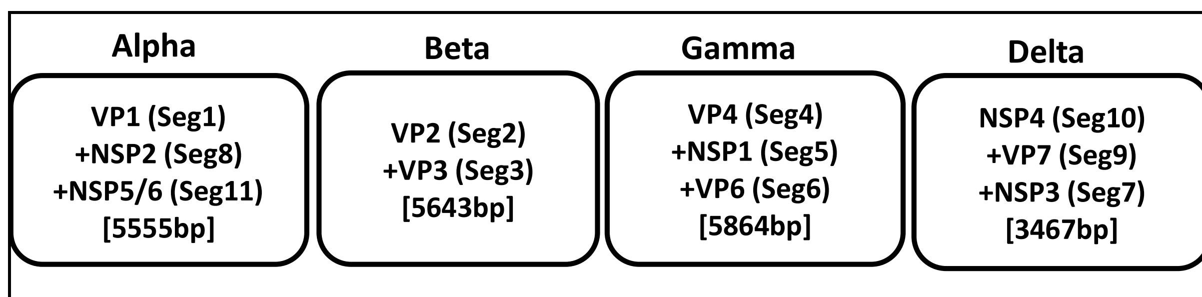

Figure 5.3: Rotavirus SA11 multiple genome segment insert plasmid composition.
Alpha-Delta contains all 11 rotavirus SA11 genome segments

Every genome segment insert was flanked by a unique blunt end restriction enzyme site. The designed inserts were synthesized and cloned into a pUC57 vector by GenScript. All four cassettes were subsequently sub-cloned into a pSMART LCAmp (Lucigen) vector. The recombinant pSMART vectors containing the synthesised genome segments were shipped from GenScript to our laboratory in lyophilised form. Following the recommendations of GenScript, the vials containing each of the lyophilised plasmids containing the rotavirus genome cassettes were centrifuged at 6 000 x g or 1 minute at 4°C to minimize loss of material during opening. The lyophilised plasmid DNA (4 µg) was then dissolved in 20 µl molecular grade water. In order to enhance the solubility of the plasmids DNA, vials were heated to 50 °C for 20 minutes.

5.2.2 MA104 Codon-optimised rotavirus SA11 expression plasmids

The codon-optimised plasmid set was designed in an attempt to produce a rotavirus replication complex scaffold. Seven codon-optimised open reading frames were designed and placed under the control of a CMV promoter. These included plasmids encoding the replication complex (VP1, VP2, VP3 and VP6), viroplasm-like structure (NSP2 and NSP5) and the innate immune response suppression protein (NSP1). Individual rotavirus SA11 genome segment were codon-optimised for expression in MA104 cells. Codon optimisation was done by OptimumGene™ Gene Design system from GenScript. This technology is designed to alter gene sequences to attain the highest probable levels of efficiency in a specific expression system. The OptimumGene™ uses an innovative algorithm which can take into consideration a wide variety of factors involved in protein expression such as mRNA structure and *cis*-elements in transcription and translation. Every individual cDNA cassette

was flanked by BsmBI restriction sites**.** An insert only contains a single codon-optimised genome segment, resulting in eight individual plasmids. The phCMVDream (***Figure 5.4***) expression plasmid was kindly provided by Professor Piet van Rijn (CVI, Lelystad, The Netherlands) (see ***Appendix D*** for phCMVDream sequence).

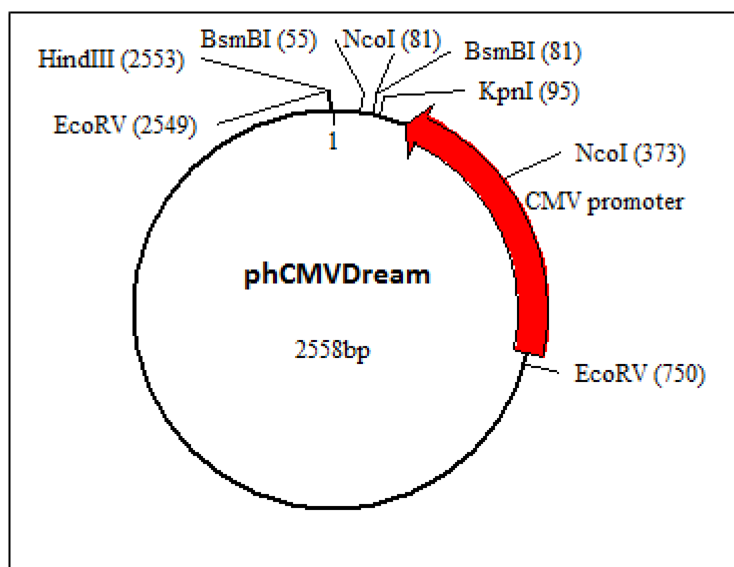

Figure 5.4: ***Plasmid map of phCMVDream.*** *Codon-optimised rotavirus SA11 open reading frames were cloned in at the BsmBI restriction enzyme digestion sites by GenScript. Plasmid map indicates restriction endonuclease sites and the red arrow the CMV promoter. Map was created using DNAMAN version 6 (Lynnon Corporation).*

The phCMVDream vector is based on the pSMART LC Amp plasmid from Lucigen. The vectors containing the synthesised genome segment inserts were shipped from GenScript to our laboratory in lyophilised form. The phCMVDream vector is based on the pSMART LC Amp plasmid from Lucigen. The vectors containing the synthesised genome segment inserts were shipped from GenScript to our laboratory in lyophilised form.

5.2.3 Transformation of ABLE C competent cells and plasmid amplification

ABLE C (Stratagene) competent cells were used to amplify the pUC57/pSMART LCamp/phCMVDream plasmids. ABLE C cells reduce the copy number of the plasmids. The

reduction of the copy number was a precautionary measure to decrease the potential toxicity of some rotavirus genome segments in the transformed cells. For the amplification of competent cells, ABLE C cells were thawed on ice and 1 µl of the stock solution inoculated in 50 ml Luria broth (LB). The culture was incubated at 37 °C overnight. Following overnight incubation, 20 ml of the culture was added to 200 ml LB and grown to an OD_{600} of 0.3 and placed on ice. Cells were pelleted, pooled and resuspended in 10% glycerol. Aliquots of 50 µl ABLE C cells were made and stored at -80 °C. For transformations, a total of 1 ng of the pUC57 plasmid containing the rotavirus genome inserts was added to 50 µl of ABLE C cells in a 1mm electroporation cuvette (BioRAD). Electroporation was performed in a GenePulser XCell (BioRAD) at 1 800V, 300Ω with a capacitance of 25 µF. Super optimal broth (SOC) was immediately added to the transformed cells and the cells were incubated at 37 °C for 1 hour (shaking vigorously at 300 rpm). After an hour, cells were plated on SOC agar plates containing 100 µg/ml ampicillin (Sigma) and incubated at 37 °C overnight. Following incubation, several transformed colonies were used to prepare glycerol stocks which were stored at -20 °C. For plasmid amplification, a single transformed colony was inoculated in a 5 ml LB medium starter culture (containing 100 µg/ml ampicillin) for each rotavirus genome insert and incubated at 37 °C (shaking vigorously at 300 rpm) for 8 hours. After 8 hours, 5 µl of this starter inoculate was transferred to 100 ml of LB media containing 100 µg/ml ampicillin and incubated at 37 °C (shaking at 220 rpm) for 16 hours.

5.2.4 Plasmid extraction and purification

Several plasmid extraction and purification methods were investigated. Finally, a modified QIAGEN Plasmid Purification technique proved to be the most effective for purifying plasmid DNA with high yields. Cultures were grown to a cell density of approximately 4×10^9 cells per milliliter, which corresponds to a cell pellet weight of approximately 3 g/L LB medium. Cells were harvested (pelleted) by centrifugation at 6000 x *g* for 15 min at 4°C. The cell pellet was then resuspended in 4 ml in the Qiagen resuspension buffer, P1. Vigorous vortexing may be required to sufficiently resuspend the pellet. Adding 0.5 µl RNase A (Qiagen) to the P1 buffer significantly increases the final plasmid yield. After the pellet was resuspended, 4 ml of the lysis buffer, P2, was added to the solution and thoroughly mixed. It is important not to vortex the solution, as this may result in extensive shearing of the

plasmid DNA. The lysis reaction was incubated for 5 min at room temperature. Care was taken not to expose the solution to the lysis reaction for longer than 5 min. After 5 min, 4 ml of buffer P3 (precipitation buffer) was added to the mixture and thoroughly mixed. Mixture was incubated on ice of 15 min. The white precipitate contains mainly cellular proteins, genomic DNA and cell debris. To remove the precipitate, mixture was centrifuged at 20 000 x g using a Surespin 630 rotor in a Sorvall ultracentrifuge at 4 °C for 60 min. The supernatant was carefully decanted and a second centrifugation at 20 000 x g at 4 °C for 15 min was performed to get rid of the excess precipitant. The supernatant should be clear after centrifugation, otherwise the centrifugation step was repeated to avoid the blockage of the binding column. The QIAGEN-tip 20 binding column was prepared by applying 10 ml QBT buffer and emptying it through gravity-flow. The decanted supernatant was then applied to the pre-treated QIAGEN-tip 20 binding column and left to empty through gravity-flow. Plasmid DNA bound to the column. The QIAGEN-tip 20 binding column was then washed twice with the wash buffer, QC. After the wash, the column was placed in a 15 ml collection tube and 5 ml of the elution buffer, QF, was applied. The eluted plasmid DNA was precipitated by adding 3.5 ml (0.7 volumes) of chilled isopropanol and immediately centrifuged at 14 000 x g for 45 min. The isopropanol was carefully decanted and the pellet was washed with 70% ice cold ethanol by centrifugation at 14 000 x g for 15 min. After 15 min the supernatant were carefully decanted and the pellet air dried. The dry pellet was resuspended in 100 – 200 µl molecular grade water that was pre-heated to 60 °C for better resuspension.

5.2.5 Transfection optimisation of rotavirus plasmids in mammalian cell cultures

In an attempt to determine the cell line best suited for rotavirus protein expression from plasmids, the MA104, BSR, COS-7 and HEK 293H cell lines were individually transfected with either the rotavirus SA11 or Wa constructs. MA104, COS-7, BSR and HEK 293H cells were cultured in serum free Dulbecco's modified essential medium (D-MEM; Hyclone), 1% penicillin/streptomycin/amphotericin B (Gibco) and 1% non-essential amino acids (Lonza). Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2.

About 16 hours before the first transfection cells were seeded in 24-well plates (NuncTM) and at approximately 80–90% confluence, the cells were transfected using XtremeGENE HP

as described in ***section 4.2.1*** (***chapter 4***). The T7 polymerase was provided by a recombinant fowlpox virus (rFPV) (Britton et al., 1996, Skinner et al., 2005). The expression of T7 RNA polymerase by the rFPV was successfully utilised in the enteropathogenic norovirus reverse genetics system (Yunus et al., 2010). Apart from providing a T7 RNA polymerase, another major advantage of the rFPV is its inability to replicate in mammalian cells and that it does not cause significant cytotoxicity (Das et al., 2000, Somogyi et al., 1993).

Cells were infected with the rFPV for an hour just prior to the first transfection. Each well of the 24-well plate was transfected with equimolar amounts of the individual genome segments by adding up to a total of 4 µg of plasmid DNA. Cells usually underwent a second transfection of 2 µg of plasmid DNA, 12 hours after the initial transfection. In order to test the transfection efficiency of the different cell lines, the expression of VP6 was determined over time using immunostaining (***Chapter 4, section 4.2.1.5***). The peGFP-N1 vector (Clonetech) was used as an expression control (***Chapter 4, section 4.2.1.1***). The immunostained cells were then visualised with an Eclipse TE2000-S microscope (Nikon) and images were captured using the NIS-Elements (2.30) software (Nikon).

5.2.6 Transfection of rotavirus SA11 transcripts into a variety of cell lines

About 16 hours before the first transfection, BSR, COS-7, HEK 293H and MA104 cells were seeded in 24-well plates (Nunc™). See ***Chapter 4, section 4.2.1.5*** for detailed procedure.

5.2.7 Transfection of codon-optimised plasmids and SA11 DLP-derived transcripts into mammalian cells

About 16 hours before the first transfection, BSR, COS-7, HEK 293H and MA104 cells were seeded in 24-well plates (Nunc™). A dual transfection strategy was followed. For the first transfection, 4 µg of the codon-optimised expression plasmids were transfected. Approximately 12 hours after the first transfection, 0.5 µg of DLP-derived rotavirus SA11 transcripts were transfected as described in ***Chapter 4, section 4.2.1.5.*** Cells were harvested by scraping 48 hours after the initial transfection and certificated at 2 000 x g to pellet

remaining cells and cellular debris. About 100 µl of the supernatant was removed from a total amount of 200 µl. Porcine trypsin IX (Sigma) (with a final concentration of 10 µg/ml) was added to the supernatant and incubated for 1 hour in a 37°C water bath. The remaining supernatant was used to resuspend the cellular pellet and 10 µg/ml porcine trypsin was also added to the resuspension and incubated for 1 hour at 37°C. The pellet was also blind passaged in order to include any viruses potentially contained in the harvested cells or cell debris. The two solutions were then applied to cells in a 6-well plate (Nunc™) containing DMEM with 1 µg/ml porcine trypsin and supplemented with 1% non-essential amino acids (Gibco) and 1% penicillin/streptomycin/amphotericin (Lonza). Cells were incubated between 96 – 120 hours at 37 °C in a humidified atmosphere of 5% CO_2. Cells were harvested after 96 – 120 (depending on cell type) by freeze-thawing and the supernatant and pellet isolated as described earlier. Approximately 75% of the trypsin treated supernatant and resuspended cellular pellet were used to blind passage the cells at least 3 times. Cells were constantly monitored for CPE and a portion of the cellular suspension of the blind passages was used for dsRNA isolation and cDNA synthesis using sequence-independent amplification (see ***section 3.2.2*** for sequence-independent cDNA synthesis method).

5.2.8 Determination of the expression of certain rotavirus SA11 transcript-derived proteins

24 hours after the transfection with rotavirus SA11 transcripts, cells were thoroughly washed with PBS and fixed as described in ***section 5.2.5***. The expression of rotavirus VP1, NSP2 and NSP5 were determined by immunofluorescence microscopy. Primary polyclonal guinea pig antibodies to VP1 and NSP2 were kindly donated by John Patton (NIH, USA) and primary guinea pig antibodies to NSP5 were a generous gift from Oscar Burrone (ICGEB, Italy). The primary antibodies were diluted 1:200 in PBS and incubated at room temperature for 2 hours. A goat pAB to guinea pig (Abcam) secondary antibody was diluted 1:200 in PBS and incubated for 1 hour at room temperature. The immunofluorescence cells were also visualised with an Eclipse TE2000-S microscope (Nikon) and images were captured using the NIS-Elements (2.30) software (Nikon). eGFP was used as an expression control. Unfortunately we are not in the possession of antibodies to test for the expression of other viral proteins.

5.3 Results and Discussion

5.3.1 Optimisation of transfection conditions of plasmids encoding rotavirus proteins and expression of the viral proteins

To determine the mammalian cell line best suited for rotavirus protein expression from plasmids, the MA104, BSR, COS-7 and HEK 293H cell lines were individually transfected with the rotavirus expression plasmid sets (encoding Wa and SA11 proteins) and the plasmids containing SA11 codon-optimised open reading frames. The expression of rotavirus VP6 was monitored over a 120 hour period in the four different cell lines (***Figure 5.5***) and eGFP (***Chapter 4, section 4.2.1.1***) was used as an expression control. This experiment was done in duplicate. The NCDV antibody to VP6 was the only commercially available antibody against any rotavirus protein available at the beginning of this study and was hence used as the primary test of expression. The expression of VP6 could be confirmed in all cell lines transfected with the rotavirus Wa and SA11 (Gamma) plasmids containing genome segment 6. It is clear that the expression of viral protein VP6 is not as high in comparison to eGFP expression, ranging from ~10 – 30% of cells expressing VP6 over a 120 hour period in different cell types. VP6 expression in BSR cells was the lowest at just over 10% of cells expressing VP6 (***Figure 5.5A***). Approximately 20% of HEK 293H and COS-7 cells expressed VP6 (***Figure 5.5B and C***). MA104 cells showed the best VP6 expression at 30% (***Figure 5.5D***). In an attempt to determine if the low expression of VP6 was a result of insufficient amount of plasmid DNA, cells were transfected with different plasmid concentrations ranging from 1 - 6 µg of the plasmid DNA (data not shown).

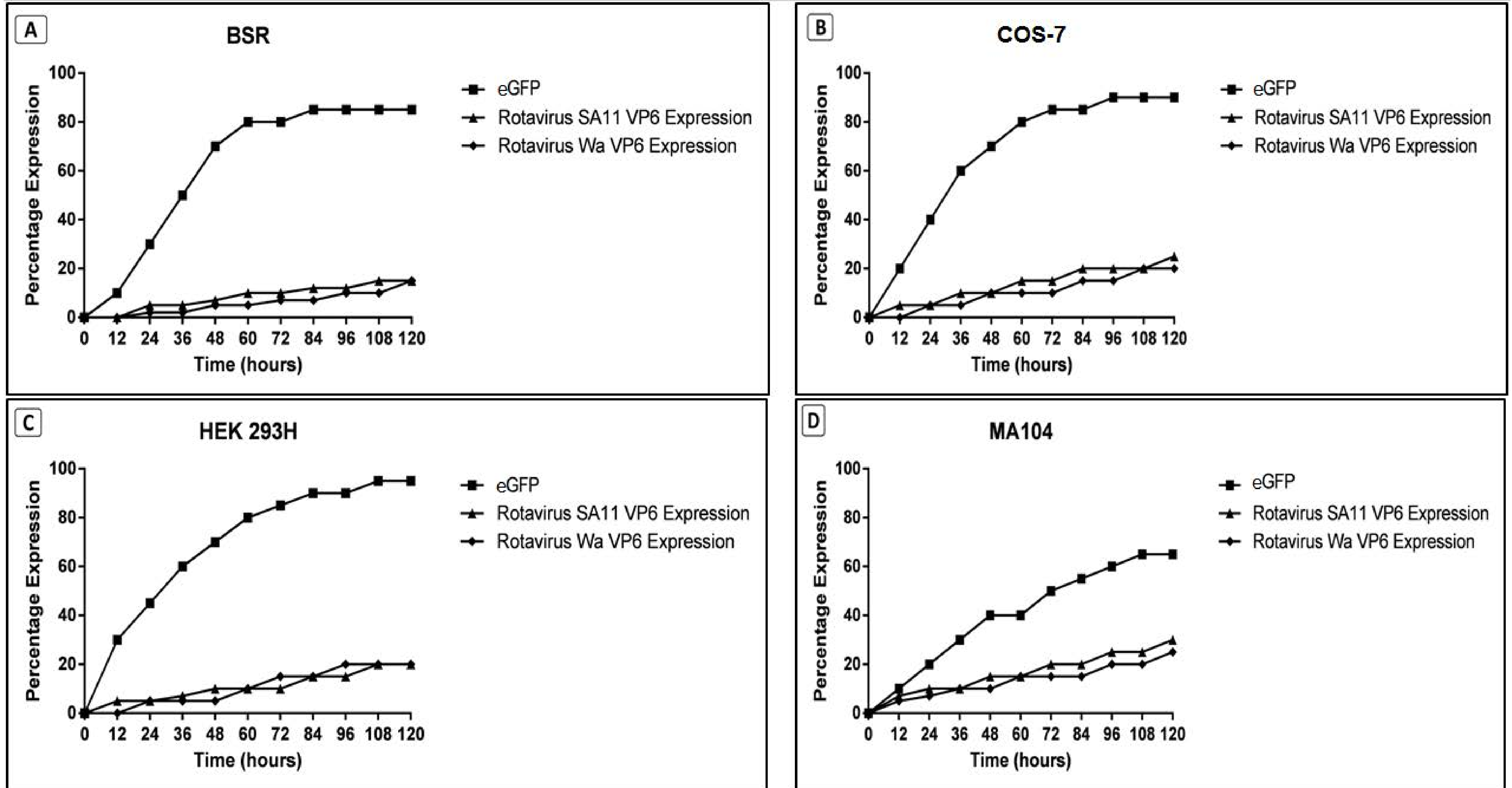

Figure 5.5: ***Comparison of the expression of rotavirus VP6 in cultured cell lines (A: BSR, B: COS-7, C: HEK and D: MA104) after transfection with SA11 and Wa expression plasmids.*** *The first set of cells was transfected with the SA11 plasmid Gamma containing the genome segment encoding for VP6. The second set of cells was transfected with the plasmid containing the rotavirus Wa genome segment 6. eGFP was used as an expression control (peGFP-N1).*

The best expression levels of VP6 were observed when transfecting with 4 – 6 µg of plasmid but expression in most cell lines was never more than 20 %. It seems that MA104 cells, apart from being difficult to transfect, are best for expression of viral proteins, or at the very least VP6. These findings correlates with the general experience that MA104 cells are more suitable for rotavirus propagation.

After extensive further experimentation and optimisation of transfection condition, VP6 expression in MA104 cells could be boosted from 30% of cells expressing VP6 to between 50-65%. Postponing the rFPV infection to 12 hours after the initial plasmid transfection and introducing a second transfection, significantly increased VP6 expression (***Figure 5.6 and Figure 5.7***).

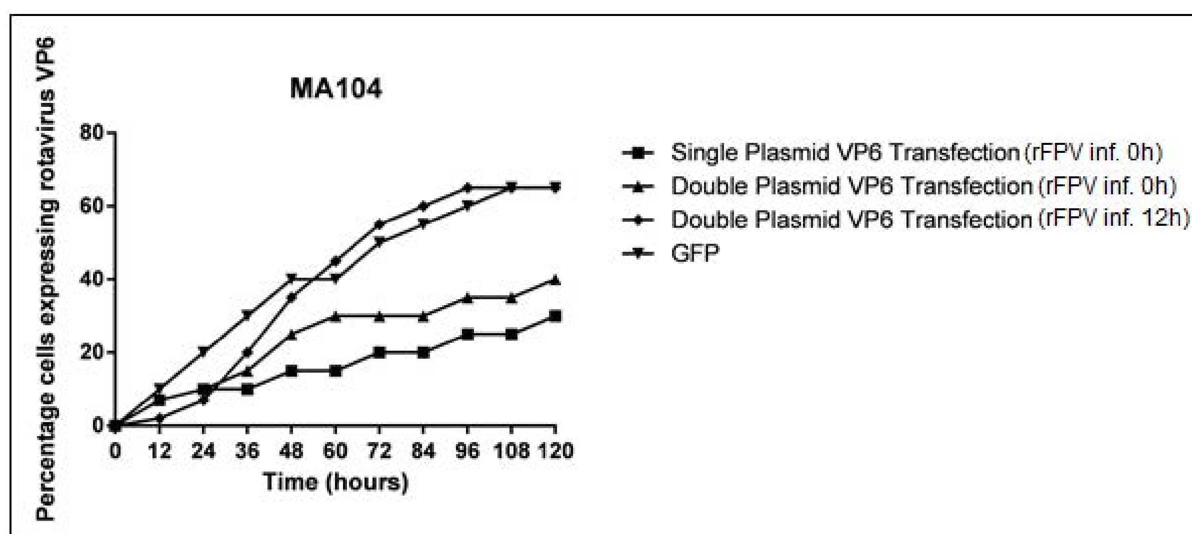

Figure 5.6: The effect of different rFPV infection times and number of plasmid transfections on the percentage of MA104 cells expressing plasmid derived rotavirus SA11 VP6.

The increase in VP6 expression when postponing the rFPV infection for 12 hours is clearly visible under a light microscope (***Figure 5.7***). In addition to delaying the rFPV infection, prolonging the rFPV exposure time to 2 hours also increased expression of VP6 (results not shown). No visible negative effects could be observed on mammalian cells after prolonged exposure to rFPV, except for HEK 293H cells in which minimal cell death were observed after 2 hours.

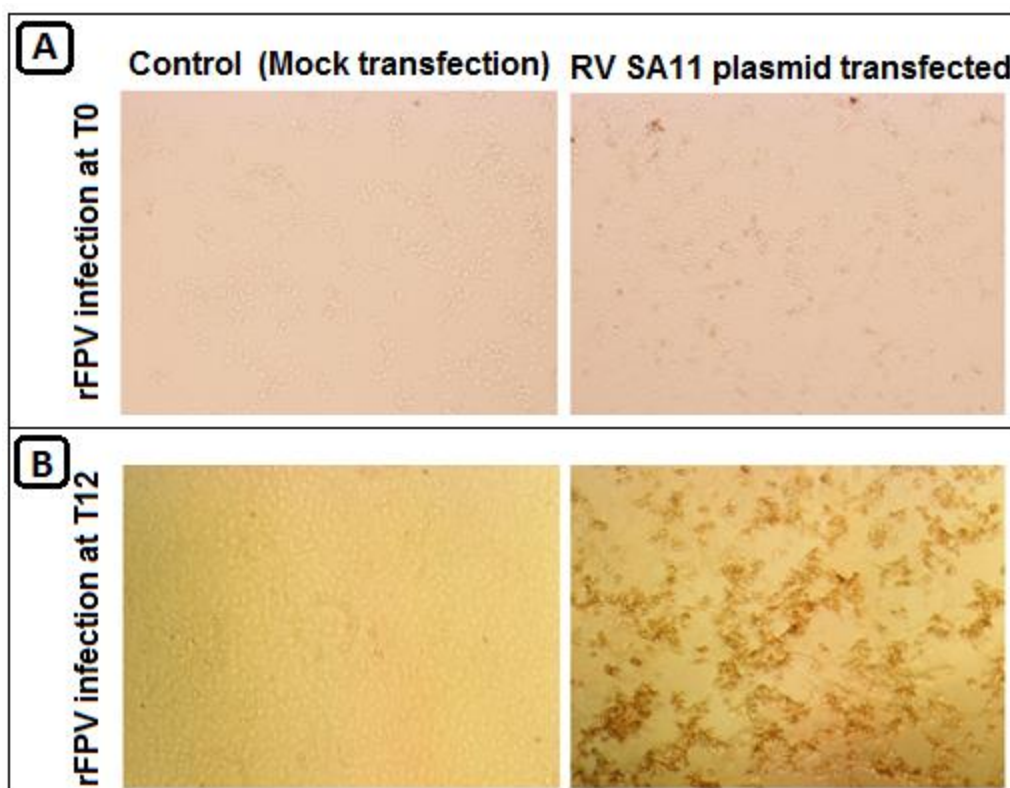

Figure 5.7: ***Comparison of the expression of plasmid derived rotavirus SA11 VP6 in MA104 cells infected with rFPV at different time.*** *(A) MA104 cells infected with rFPV an hour before the first plasmid transfection. (B) MA104 cells transfected 12 hours after the first plasmid transfection. The control was a mock transfection.*

Infecting the cells with rFPV 12 hours after the first transfection dramatically increased the expression of VP6 from about 30% MA104 cells expressing rotavirus VP6 to 65% (***Figure 5.6****)*. Transfecting with a total amount of 4 µg DNA yielded the best VP6 expression without negatively affecting cell viability. Dual transfections significantly increased VP6 expression with the best expression obtained when spacing out the second transfection between 16 – 20 hours after the first transfection. The second transfection was performed with half of the amount of plasmid DNA of the first transfection (~2 µg). More than two transfections have a negative effect cell viability, this is especially true for more delicate cell lines such as HEK 293H and BSR cells. It became clear that each cell line has a unique set of transfection parameters and is best to standardize transfection conditions individually. ***Figure 5.8*** shows

the optimised expression of rotavirus VP6 in MA104 cells after 60 hours. All subsequent plasmid transfections were performed taking these optimised conditions into account.

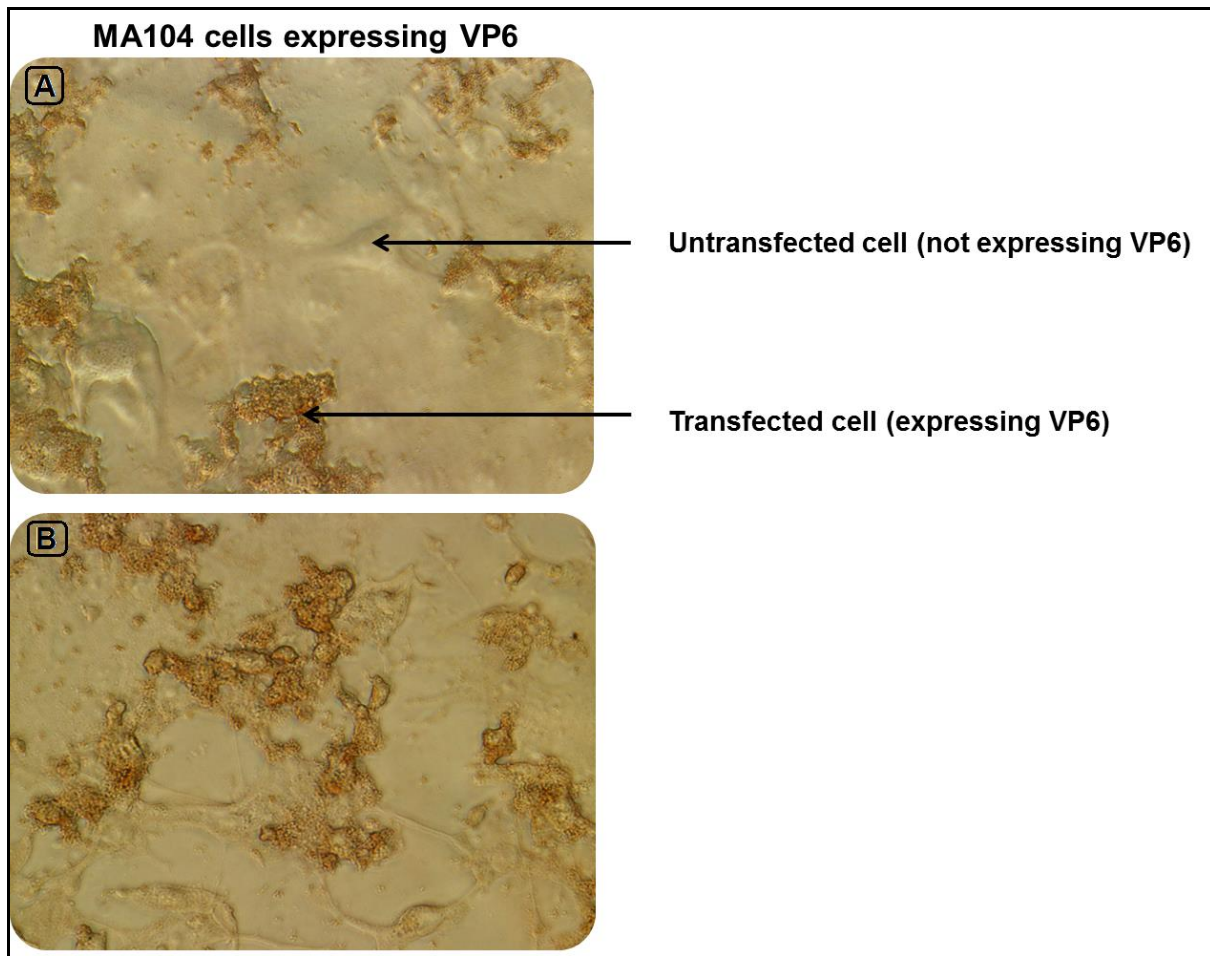

Figure 5.8: Immunostaining of MA104 cells expressing VP6 after transfection optimisation. *(A) MA104 cells transfected with 4 µg of equimolar amounts of all 11 rotavirus Wa expression plasmids followed by a second transfection of 2 µg of plasmid DNA. (B) MA104 cells transfected with 4 µg of equimolar amounts of rotavirus SA11 expression plasmids followed by a second transfection of 2 µg of plasmid DNA. 40 x magnifications, unfiltered.*

The promising expression obtained for VP6 prompted the search for antibodies against other rotavirus proteins to assist in the detection of these proteins. Therefore, the expression of rotavirus VP1, NSP2 and NSP5 could also be determined by immunofluorescence microscopy (***Figure 5.9***).

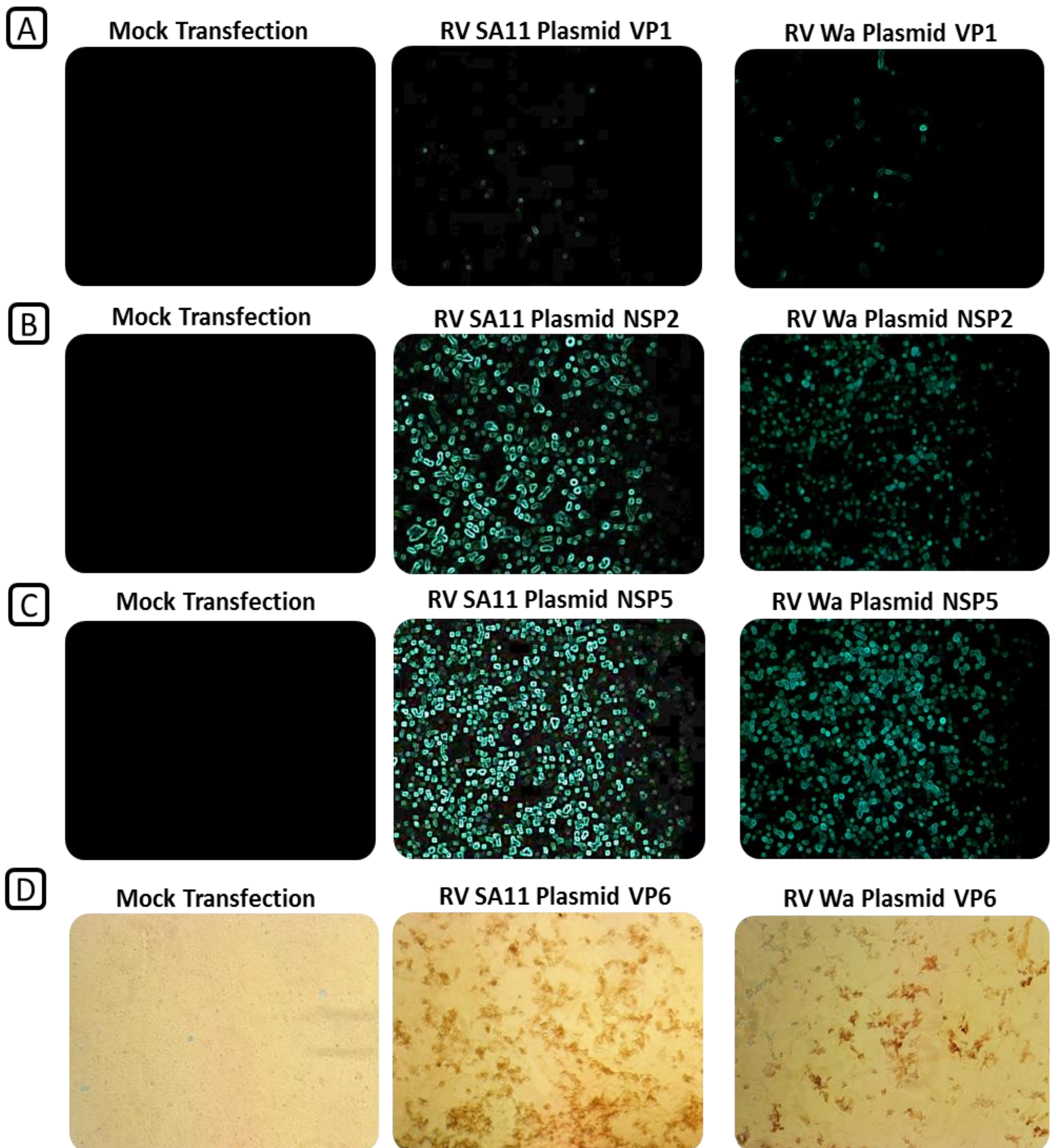

Figure 5.9: ***Immunological detection of plasmid derived rotavirus protein expression in MA104 cells.*** *Cells were immunostained with antibodies showing the detection of rotavirus proteins, (A) VP1, (B) NSP2, (C) NSP5 and (D) VP6 in MA104 cells following the transfection of the rotavirus SA11 or Wa expression plasmids. The expression of rotavirus VP1, NSP2 and NSP5 were determined by immunofluorescence microscopy. The expression rotavirus VP6 was determined by immunostaining. Brightness and contrast of picture sets A, B and C enhanced in MS PowerPiont Professional Plus 2010.*

The expression of VP1 is noticeably lower than that of the other viral proteins examined and further optimisation is needed. The plasmid-based expression of VP1, NSP2 and NSP5 was not determined in BSR and COS-7 cells. Since the expression of 4 for the 11 rotavirus proteins could be confirmed, it was assumed that expression of the other proteins was also obtained.

These plasmid sets proved to be competent expression vectors even though they cannot support the generation of (+) ssRNA with the exact 3’ ends needed for a reverse genetic system. During transfection optimisation experiments, it was obseved that viability of cultured cells transfected with rotavirus Wa plasmids were marginally lower than observed in the SA11 plasmid sets. To examine what the effect of transfecting each individual rotavirus Wa expression plasmid (each containing a single genome segment) has on cell viability, MA104, COS-7, BSR and HEK 293H cells were transfected with 4 µg of each rotavirus Wa expression plasmid and cell viability was monitored for 120 hours (the results are defined in ***Table 5.1***).

Table 5.1: The effect of transfecting plasmids containing individual rotavirus Wa genome segments on MA104, COS-7, BSR and HEK 293H cells over a 120 hour period

RV-Wa Genome Segment	GS-1					GS-2					GS-3					GS-4				
Encoded Viral Protein	VP1					VP2					VP3					VP4				
	24h	48h	72h	96h	120h	24h	48h	72h	96h	120h	24h	48h	72h	96h	120h	24h	48h	72h	96h	120h
CPE on **MA104** cells	-	-	-	-	-	-	-	-	-	-	-	+	++	+++	∞	-	-	+	++	++
CPE on **COS-7** cells	-	-	-	-	+	-	-	-	+	+	-	+	++	+++	∞	-	+	++	++	+++
CPE on **BSR** cells	-	-	-	-	+	-	-	-	+	++	-	+	+++	∞	∞	-	+	++	++	+++
CPE on **HEK 293H** cells	-	-	-	-	+	-	-	-	-	+	-	+	++	+++	∞	-	+	+	++	++
Control	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+

RV-Wa Genome Segment	GS-5					GS-6					GS-7					GS-8				
Encoded Viral Protein	NSP1					VP6					NSP3					NSP2				
	24h	48h	72h	96h	120h	24h	48h	72h	96h	120h	24h	48h	72h	96h	120h	24h	48h	72h	96h	120h
CPE on **MA104** cells	-	++	+++	∞	∞	-	-	+	++	++	-	++	+++	∞	∞	-	-	+	++	++
CPE on **COS-7** cells	-	++	+++	+++	∞	-	+	+	++	++	-	++	+++	+++	∞	-	-	+	++	++
CPE on **BSR** cells	-	++	+++	∞	∞	-	+	+	++	++	-	++	+++	∞	∞	-	+	+	++	++
CPE on **HEK 293H** cells	-	++	+++	∞	∞	-	+	+	++	++	-	++	+++	∞	∞	-	+	+	++	++
Control	-	-	-	-	+	-	-	-	+	++	-	-	-	-	+	-	-	-	+	++

RV-Wa Genome Segment	GS-9					GS-10				GS-11			
Encoded Viral Protein	VP7					NSP4				NSP5/6			
	24h	48h	72h	96h	120h	48h	72h	96h	120h	48h	72h	96h	120h
CPE on **MA104** cells	-	-	+	++	+++	+	++	+++	∞	++	+++	∞	∞
CPE on **COS-7** cells	-	-	+	++	+++	++	+++	∞	∞	+	++	+++	∞
CPE on **BSR** cells	-	-	+	++	+++	+	++	+++	∞	++	+++	∞	∞
CPE on **HEK 293H** cells	-	-	+	++	+++	++	+++	∞	∞	++	+++	∞	∞
Control	-	-	+	+	-	-	-	+	-	-	+	++	

4 µg of each expression plasmid containing rotavirus Wa genome segments was used for transfection per well of a 6-well plate
Control: A mock transfection was used as a control and maintained in serum free media containing non-essential amino acids and antibiotics
The XtremeGENE transfection mixture was removed from the cells after 24 hours and replaced with serum free media containing non-essential amino acids and antibiotics
- indicates no/very little cell death, + indicates 10-20% cell death, ++ indicates 20-40% cell death, +++ indicates 40-60% cell death and ∞ indicates mass cell death, CPE was gauged by visual inspection
Genome segment or expressed viral protein has no or very little observed negative effects on the cellular viability (0-20% cell death), **Genome segment or expressed viral protein had moderate negative effects on the cellular viability after 120 hours (20-60% cell death)** and **Genome segment or expressed viral protein caused total cell death after 120 hours (100% cell death)**

Transfecting plasmids encoding VP1 and VP2 had no or very little observable effect on the cell viability of MA104, COS-7 and HEK 293H cells within a 120 hour period. Immunological detection showed low expression levels of plasmid derived rotavirus VP1 (***Figure 5.9***) and expression of this relatively large viral protein may be too slow to have a noticeable effect on cellular viability. Transfecting plasmids encoding for VP4, VP6, NSP2 and VP7 had a moderate cytotoxic effect on MA104 and HEK 293H cells. Plasmids encoding for the capping structure, VP3, induced mass cell death within 120 hours in all cell types examined. The innate response suppressing protein, NSP1, also led to mass cells death 96 hours after transfection of MA104, BSR, COS-7 and HEK 293H cells. The transfection of plasmids encoding NSP3 proved to be one of the most cytotoxic genome segments transfected. This non-structural protein is involved in translational regulation and host cell shut-off (Montero et al, 2006). Prolonged exposure to this viral protein in isolation may have negative effects on normal host cell translation. Transfecting the plasmid encoding NSP4 also caused mass cell death within 120 hours and it is known to be an enterotoxin (Ball et al., 1996, Horie et al., 1999, Tian et al., 1996). The innate response to the expressed rotavirus proteins will be presented in ***Chapter 6.***

5.3.2 Standardisation of transfection conditions for rotavirus transcripts

A variety of cell lines were used to standardise the transfection conditions of rotavirus transcripts. The transfection reagent XtremeGENE HP (Roche) proved to be best for transfecting rotavirus transcripts (***Chapter 4***). Initially, the same transfection procedure was employed as for the plasmid sets by transfecting 4 µg of SA11 transcripts in a 24-well plate. In the case of rotavirus transcripts, this resulted in mass cell death within 5 hours after transfection. Mlera transfected 1-1.5 µg of DLP derived transcripts (Mlera, 2013). In the current study, mass cell death was observed within 12 hours after transfecting of rotavirus SA11 transcripts based on the amount of transcripts transfected (1-1.5 µg) by Mlera. It must be noted that the current study employed a heavily modified transfection procedure and the accelerated cell death observed might be due to a higher transfection efficiency of the modified X-tremeGENE HP procedure. After extensive optimisation (optimisation results not shown), the following parameters proved to be the best suited for rotavirus transcript transfections using the modified transfection procedure: The

transfection of 0.5 µg of rotavirus transcripts per well (24-well plate, Nunc^{TM}) resulted in approximately the same level of VP6 expression as 4 µg (***Figure 5.10***) of plasmid DNA without eliciting a fatal cellular innate immune response within 20 hours after transfection.

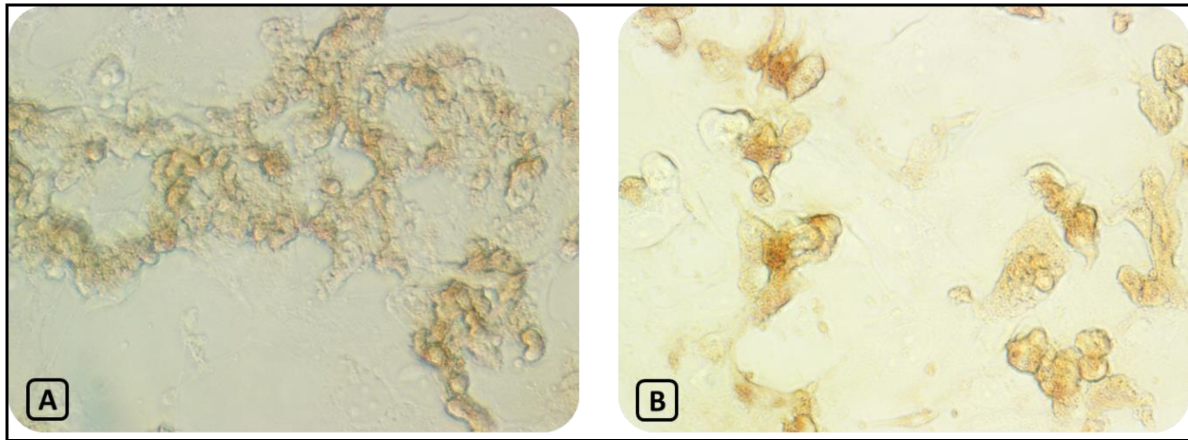

Figure 5.10: ***Immunostaining of rotavirus SA11 VP6 of MA104 cells after the transfection of (A) 4 µg rotavirus SA11 VP6 containing plasmid DNA and (B) 0.5 µg of DLP derived SA11 VP6 transcripts.***

Matsuo and Roy showed that a second transfection of bluetongue virus transcripts enhanced the rescue of this virus (Matsuo and Roy, 2009). A second rotavirus transcript transfection resulted in excelled cell death and did not seem to increase the expression of viral proteins (results not shown). Attempts were made to transfect a significant lower amount of transcripts in order to minimize cytotoxicity and enable multiple transfections were in vain (even with transcript amounts as low as 0.1 µg cell death still occurred). Expression of VP6 was observed in all cell types transfected with rotavirus transcripts (***Table 5.2***). After optimisation, the most efficient VP6 expression (40 - 60% of cells expressed VP6) was observed in MA104 cells and these cells also survived the longest period (30 – 40 hours) after transfection of transcripts. BSR and HEK 293H cells also showed relatively good VP6 expression (20- 40%).

Table 5.2: Evaluation of cell death following transfection with SA11 transcripts (0.5 µg per well of a 24-well plate) and level of VP6 expression detected

Cell line	Effect on cells	VP6 expression after 20h
MA104	Total cell death after 30-40 h	+++
BSR	Total cell death after 20-24 h	++
COS-7	Total cell death after 20-30 h	+
HEK 293H	Total cell death after 24-30 h	++

+ 0-20% of cells expressing VP6, ++ 20-40% of cells expressing VP6, +++ 40-60% of cells expressing VP6. CPE was gauged by visual inspection

The lowest transcript-derived VP6 expression was observed in COS-7 cells (< 20%). Additionally, the expression of VP1, NSP2, NSP5 and VP6 could also be shown in MA104 and HEK 293H cells (***see Figure 5.11 for expression in HEK 293H cells***).

In summary, to optimize the transfection conditions of rotavirus transcripts, a variety of mammalian cultured cell lines were used (MA104, COS-7, BSR and HEK 293H). MA104 cells proved to be the best suited for the expression of rotavirus transcripts (***Table 5.2***) and these cells could also be exposed to the rotavirus transcripts for the longest period before complete cell death was observed. In addition, the expression of transcript-derived rotavirus VP1, VP6, NSP2 and NSP5 could be confirmed with immunofluorescence or immunostaining in MA104 and HEK 293H cells. The expression of VP1 is visibly inferior to that of NSP2 or NSP5.

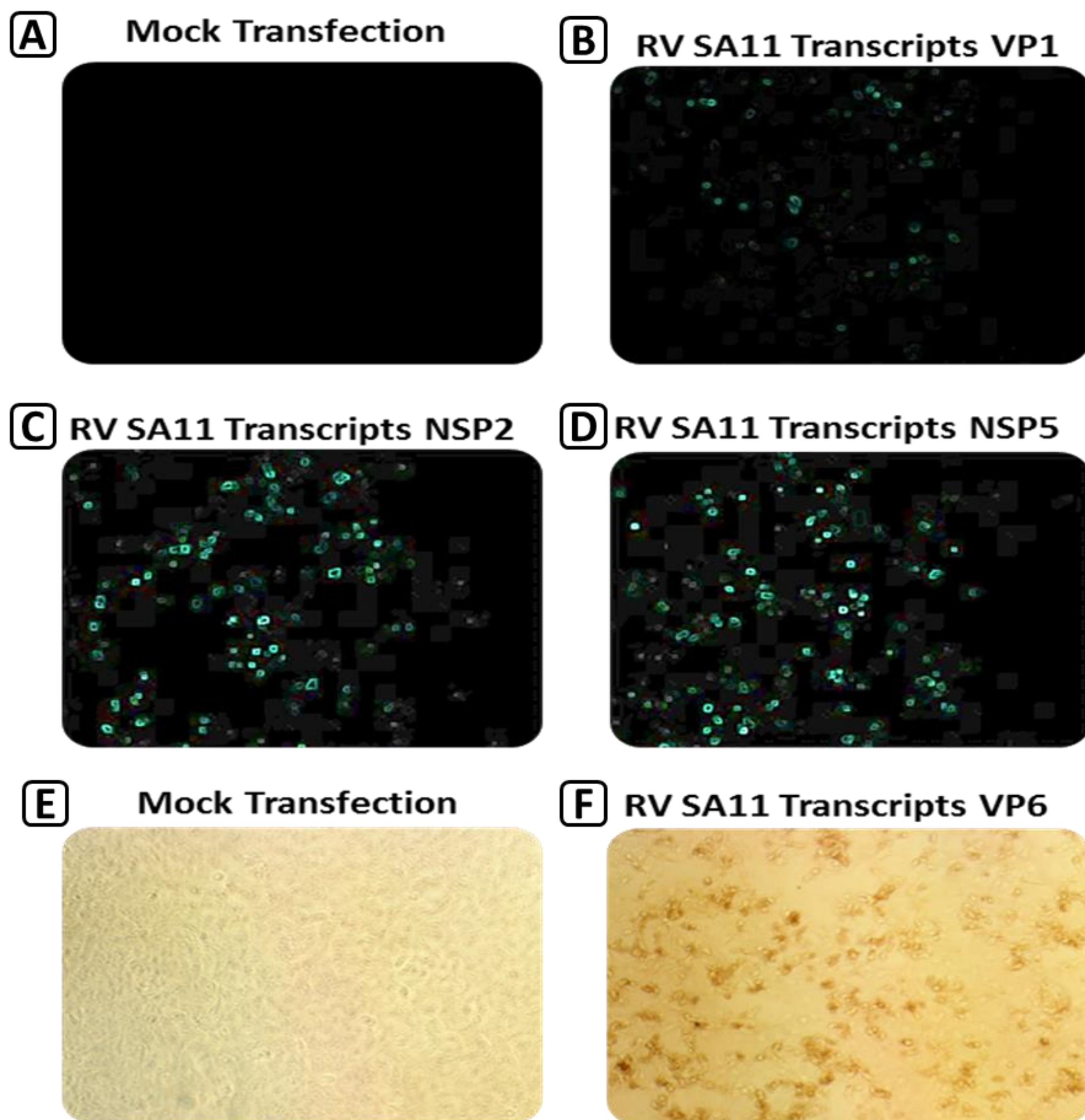

Figure 5.11: ***Immunological detection of rotavirus protein expression derived from rotavirus transcripts in HEK 293H cultured cells.*** *(A-D) Cells were immunostained with antibodies showing the detection of rotavirus proteins in HEK 293H cells following the in vitro derived rotavirus SA11 transcripts. The expression of rotavirus VP1, NSP2 and NSP5 was determined by immunofluorescence microscopy. (E-F) The expression of rotavirus VP6 was determined by immunostaining. Brightness and contrast of picture sets A, B and C enhanced in MS PowerPiont Professional Plus 2010. For original immunostaining photos and other visualisations please see* ***Appendix E****.*

5.3.3 Transfection of DLP-derived rotavirus SA11 transcripts into a variety of mammalian cells in an attempt to recover viable virus

In an attempt to recover viable rotaviruses, a wide range of mammalian cell cultures were transfected with DLP-derived rotavirus SA11 transcripts using the optimized transfection conditions. Numerous pilot transfections were performed in MA104, HEK 293H, BSR, COS-7 and co-cultured MA104/HEK 293H, MA104/BSR or MA104/COS-7 cells under various conditions (***Table 5.3).***

Two sets of experiments were performed on each cell line or co-culture by either carrying out a single or double transfection. A double transfection was performed by transfecting a second batch of rotavirus transcripts (0.5 µg) 10 hours after the initial transfection. The transfected cells were harvested between 10-48 hours after the last transfection and blind passaged at least 3 times on MA104 cells. The expression of VP6 was monitored in all cell cultures before blind passaging. Additionally, the expression of rotavirus SA11 VP1, NSP2 and NSP5 could also be shown by immunofluorescence in MA104 and HEK 293H cells. MA104 cell culture was chosen for blind passaging since SA11 propagates well in this cell line. Aliquots were taken from each blind passage, dsRNA was isolated from the culture. After transfecting a wide variety of cell cultures with SA11 transcripts under several different transfection conditions, no viable virus could be rescued after several blind passages. No viral dsRNA could be isolated or cDNA amplified using sequence-independent cDNA synthesis and amplification.

Table 5.3: Summary of transfection experiments in different mammalian cultured cells with rotavirus SA11 DLP- derived transcripts

		Transfection of rotavirus SA11 *in vitro* transcripts	
MA104	**Total amount transfected**	0.5 µg	0.5 µg
	No. of transfections	1	2
	Hours transfection complex left on cells	30-48 h	24-30 h
	No. of blind passages on MA104 cells	3	3
	Hours incubated per blind passage	120 h	120 h
	Recovered viable rotavirus	No	No
	VP6 expression detected	Yes	Yes
	Expression of other viral proteins detected	VP1, NSP2 and NSP5	*Not tested*
BSR	**Total amount transfected**	0.5 µg	0.5 µg
	No. of transfections	1	2
	Hours transfection complex left on cells	24-30 h	10 – 20 h
	No. of blind passages on MA104 cells	3	3
	Hours incubated per blind passage	96 h	96 h
	Recovered viable rotavirus	No	No
	VP6 expression detected	Yes	Yes
	Expression of other viral proteins detected	*Not tested*	*Not tested*
HEK 293H	**Total amount transfected**	0.5 µg	0.5 µg
	No. of transfections	1	2
	Hours transfection complex left on cells	24-30 h	10 – 20 h
	No. of blind passages on MA104 cells	3	3
	Hours incubated per blind passage	96 h	96 h
	Recovered viable rotavirus	No	No
	VP6 expression detected	Yes	Yes
	Expression of other viral proteins detected	VP1, NSP2 and NSP5	*Not tested*
COS-7	**Total amount transfected**	0.5 µg	0.5 µg
	No. of transfections	1	2
	Hours transfection complex left on cells	24-30 h	10 – 20 h
	No. of blind passages on MA104 cells	3	3
	Hours incubated per blind passage	120 h	120 h
	Recovered viable rotavirus	No	No
	VP6 expression detected	Yes	Yes
	Expression of other viral proteins detected	*Not tested*	*Not tested*
MA104/BSR co-culture	**Total amount transfected**	0.5 µg	0.5 µg
	No. of transfections	1	2
	Hours transfection complex left on cells	24-30 h	24-30 h
	No. of blind passages on MA104 cells	3	3
	Hours incubated per blind passage	96 h	96 h
	Recovered viable rotavirus	No	No
	VP6 expression detected	Yes	Yes
	Expression of other viral proteins detected	*Not tested*	*Not tested*
MA104/HEK 283H co-culture	**Total amount transfected**	0.5 µg	0.5 µg
	No. of transfections	1	2
	Hours transfection complex left on cells	24-30 h	24-30 h
	No. of blind passages on MA104 cells	3	3
	Hours incubated per blind passage	96 h	96 h
	Recovered viable rotavirus	No	No
	VP6 expression detected	Yes	Yes
	Expression of other viral proteins detected	*Not tested*	*Not tested*
MA104/COS-7 co-culture	**Total amount transfected**	0.5 µg	0.5 µg
	No. of transfections	1	2
	Hours transfection complex left on cells	24-30 h	24-30 h
	No. of blind passages on MA104 cells	3	3
	Hours incubated per blind passage	96 h	96 h
	Recovered viable rotavirus	No	No
	VP6 expression detected	Yes	Yes
	Expression of other viral proteins detected	*Not tested*	*Not tested*

5.3.4 Transfection of DLP-derived rotavirus SA11 transcripts in the presence of pre-expressed plasmid derived rotavirus proteins

A codon-optimised plasmid set, for the expression in MA104 cells, was designed in an attempt to produce a rotavirus replication complex scaffold**.** It was postulated that when transfecting rotavirus transcripts in the presence of core replication complexes, transcripts may be recruited into the existing cores. This is known as the core-filling model and was proposed by McDonald and Patton (McDonald and Patton, 2011). Imported transcripts would no longer be exposed in the cytoplasm for extended periods and replication would be theoretically streamlined. Individual rotavirus SA11 genome segments (VP1, VP2, VP3, VP6, NSP1, NSP2 and NSP5) were codon-optimised for the expression in MA104 cells and cloned into the phCMVDream vector (***section 5.2.1***). An insert contained a single codon-optimised open reading frame (ORF), resulting in seven individual plasmids. Each open reading frame was codon-optimized to ensure the highest probable levels of expression in MA104 cultured cells. The eight codon-optimised inserts were designed and placed under the control of a CMV promoter. MA104 and HEK 393H cell lines were individually transfected with the rotavirus SA11 codon-optimised plasmid sets. About 16 hours before the first transfection cells were seeded in 24-well plates (Nunc™) and at approximately 80–90% confluence, the cells were transfected using XtremeGENE HP as described in ***section 4.2.1.*** Because the codon-optimised system is under the control of a CMV promoter, it is independent from the rFPV infection required in the T7 expression plasmid sets. In some cases, cells underwent a second transfection, 12 hours after the initial transfection, of 2 µg of codon-optimised plasmid DNA. A second transfection of plasmid DNA did not noticeably improve viral protein expression but negatively affected cell viability and was ceased. To test the expression efficiency of the codon-optimised plasmids, the expression of VP6 and NSP2 of the codon-optimised plasmids were compared to that of the T7 rotavirus SA11 plasmids (***Figure 5.12***).

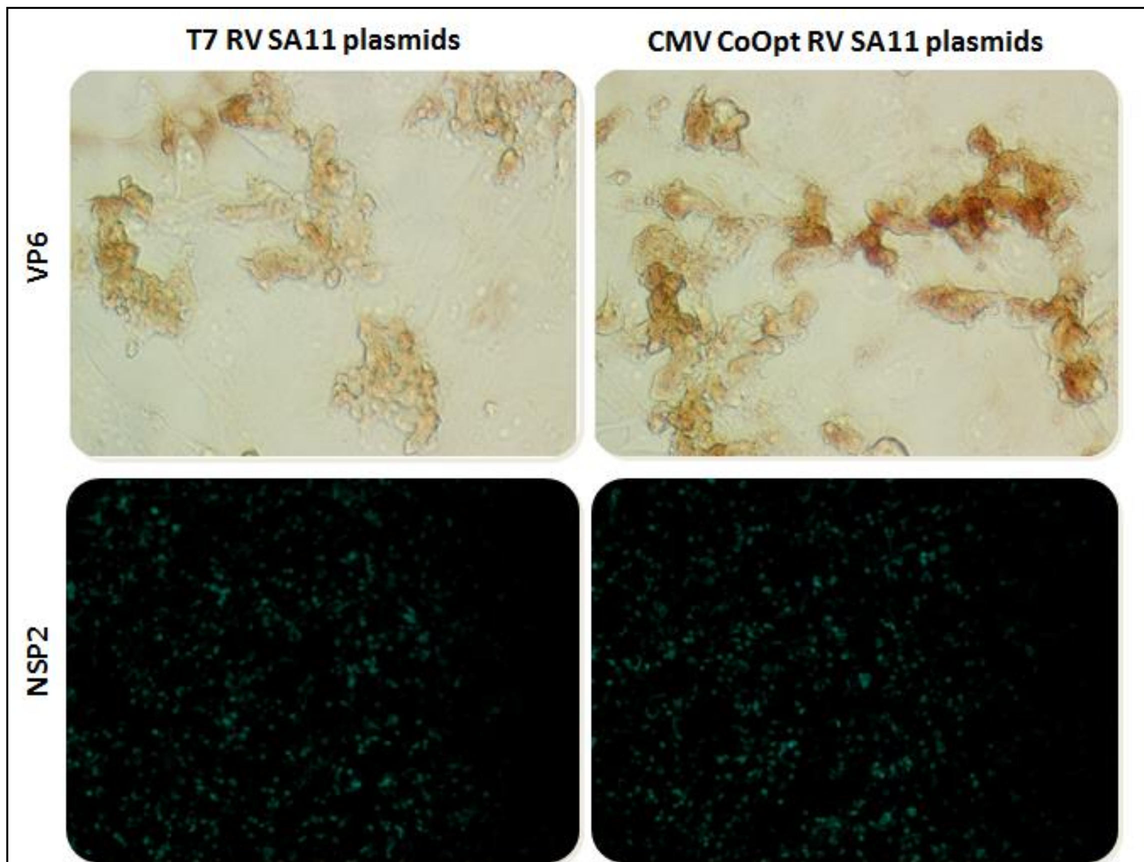

Figure 5.12: Comparison of VP6 and NSP2 expression between the CMV controlled MA014 codon-optimised rotavirus SA11 plasmids and the T7 controlled rotavirus SA11 plasmids.

The expression of codon-optimised rotavirus SA11 plasmid derived VP6 appeared marginally better than that of the T7 controlled rotavirus SA11 plasmids. A slightly larger fraction of MA104 cells expressed VP6 and it seemed as if a larger amount of VP6 was expressed per cell judged by the intensity of immunostaining. No noticeable difference was observed in the expression of NSP2.

The transfection of 0.5 µg of rotavirus SA11 transcripts was performed 12 hours after the first transfection of codon-optimised plasmids as described in ***section 5.2.10.*** Interestingly, the strong, supposed, apoptotic reaction observed with the transfection of rotavirus transcripts was drastically reduced when plasmids containing rotavirus genome segments or open reading frames were expressed beforehand. Unfortunately, no virus could be rescued after 3 blind passages. Different transfection combinations (using both the codon-optimised SA11 plasmid set and the T7 Wa and SA11 plasmid sets in combination with SA11

transcripts) were investigated (***Table 5.4, Table 5.5 and Table 5.6***). CPE was estimated using a light microscope and experiments were at least done in duplicate.

Table 5.4: MA104 cells transfected with different combinations of rotavirus SA11 DLP-derived transcripts and rotavirus Wa genome segment encoding plasmids

	1st Transfection (transfection time)	2nd Transfection (transfection time)	Viable rotavirus rescued	VP6 expression		Effect on MA104 cell viability			
					T_0	T_{12}	T_{24}	T_{36}	T_{48}
1	SA11 transcripts 0.5 µg (T_0)	None done	*No*	++	-	••	••••	∞	∞
2	RV-Wa GS 1-11 4 µg (All GS) (T_0)	SA11 transcripts 0.5 µg (T_{12})	*No*	+++	-	-	••	•••	••••
3	SA11 transcripts 0.5 µg (T_0)	RV-Wa GS 1-11 4 µg (All GS) (T_{12})	*No*	++	-	••	••••	∞	∞
4	RV-Wa GS1, 2, 3 and 6 (VP1, VP2, VP3, VP6) 4 µg (Core) (T_0)	SA11 transcripts 0.5 µg (T_{12})	*No*	+++	-	-	••	•••	••••
5	SA11 transcripts 0.5 µg (T_0)	RV-Wa GS1, 2, 3 and 6 (VP1, VP2, VP3, VP6) 4 µg (Core) (T_{12})	*No*	++	-	••	••••	∞	∞
6	RV-Wa GS 1 (VP1) 4 µg (T_0)	SA11 transcripts 0.5 µg (T_{12})	*No*	++	-	-	•••	••••	∞
7	RV-Wa GS 5 (NSP1) 4 µg (T_0)	SA11 transcripts 0.5 µg (T_{12})	*No*	++	-	-	••	•••	∞
8	SA11 transcripts 0.5 µg (T_0)	RV-Wa GS 5 (NSP1) 4 µg (T_{12})	*No*	++	-	••	••••	∞	∞
9	RV-Wa GS 6 (VP6) 4 µg (T_0)	RV-Wa GS 5 (NSP1) 4 µg (T_{12})	*No*	+++	-	•	•••	••••	∞
10	RV-Wa GS 8 and 11 (NSP2, NSP5/6) 4 µg (Viroplasm-like structure) (T_0)	SA11 transcripts 0.5 µg (T_{12})	*No*	++	-	-	••	•••	••••
11	SA11 transcripts 0.5 µg (T_0)	RV-Wa GS 8 and 11 (NSP2, NSP5/6) 4 µg (Viroplasm-like structure)(T_{12})	*No*	++	-	••	••••	∞	∞

Colour code: **Mass cell death within 36 hours after the first transfection**
Mass cell death was only observed after 48 hours following the first transfection

+ 0-10% of cells expressing VP6; **++** 10-20% of cells expressing VP6; **+++** 20-40% of cells expressing VP6. - no CPE; • 10-20% CPE; •• 20-30% CPE; ••• 30-60% CPE •••• >60% CPE; ∞ complete CPE. CPE was gauged by visual inspection

Table 5.5: MA104 cells transfected with different combinations of rotavirus SA11 DLP-derived transcripts and rotavirus SA11 genome segment encoding plasmids

	1st Transfection (transfection time)	2nd Transfection (transfection time)	Viable rotavirus rescued	VP6 expression	Effect on MA104 cell viability				
					T_0	T_{12}	T_{24}	T_{36}	T_{48}
1	RV-SA11 GS 1-11 4 µg (Alpha - Delta) (T_0)	SA11 transcripts 0.5 µg (T_{12})	*No*	+++	-	-	••	•••	••••
2	SA11 transcripts 0.5 µg (T_0)	RV-SA11 GS 1-11 4 µg (Alpha - Delta) (T_{12})	*No*	++	-	••	••••	∞	∞
3	RV-SA11 GS 1, 8, 11 4 µg (Alpha) (T_0)	SA11 transcripts 0.5 µg (T_{12})	*No*	++	-	••	•••	••••	∞
4	SA11 transcripts 0.5 µg (T_0)	RV-SA11 GS 1, 5, 11 4 µg (Alpha) (T_{12})	*No*	++	-	••	••••	∞	∞
5	RV-SA11 GS 2,3 4 µg (Beta) (T_0)	SA11 transcripts 0.5 µg (T_{12})	*No*	++	-	-	••	•••	••••
6	SA11 transcripts 0.5 µg (T_0)	RV-SA11 GS 2,3 4 µg (Beta) (T_{12})	*No*	++	-	-	•••	••••	∞
7	RV-SA11 GS 4, 5, 6 4 µg (Gamma) (T_0)	SA11 transcripts 0.5 µg (T_{12})	*No*	+++	-	-	••	•••	••••
8	SA11 transcripts 0.5 µg (T_0)	RV-SA11 GS 4, 5, 6 4 µg (Gamma) (T_{12})	*No*	++	-	••	••••	∞	∞
9	RV-SA11 GS 7, 9, 10 4 µg (Delta) (T_0)	SA11 transcripts 0.5 µg (T_{12})	*No*	++	-	••	••••	∞	∞
10	SA11 transcripts 0.5 µg (T_0)	RV-SA11 GS 7, 9, 10 4 µg (Delta) (T_{12})	*No*	++	-	••	••••	∞	∞

Colour code: **Mass cell death within 36 hours after the first transfection**
Mass cell death was only observed after 48 hours following the first transfection

+ 0-10% of cells expressing VP6, ++ 10-20% of cells expressing VP6, +++ 20-40% of cells expressing VP6

- no CPE; • 10-20% CPE; •• 20-30% CPE; ••• >50% CPE; ∞ complete CPE. CPE was gauged by visual inspection.

Table 5.6: MA104 cells transfected with different combinations of rotavirus SA11 DLP-derived transcripts and rotavirus SA11 codon-optimised genome segment encoding plasmids

	1st Transfection (transfection time)	2nd Transfection (transfection time)	Viable rotavirus rescued	VP6 expression	Effect on MA104 cell viability				
					T_0	T_{12}	T_{24}	T_{36}	T_{48}
1	RV-SA11 CoOpt GS 1, 2, 3, 5, 6, 8 and 11 4 µg (All CoOpt SA11 plasmids) (T_0)	SA11 transcripts 0.5 µg (T_{12})	*No*	+++	-	-	••	•••	••••
2	SA11 transcripts 0.5 µg (T_0)	SA11 CoOpt GS 1, 2, 3, 5, 6, 8 and 11 4 µg (All CoOpt SA11 plasmids) (T_{12})	*No*	++	-	••	••••	∞	∞

Colour code: Mass cell death within 36 hours after the first transfection
Mass cell death was only observed after 48 hours following the first transfection
+ 0-10% of cells expressing VP6, ++ 10-20% of cells expressing VP6, +++ 20-40% of cells expressing VP6
- no CPE; • 10-20% CPE; •• 20-30% CPE; ••• >50% CPE; ∞ complete CPE. CPE was gauged by visual inspection

MA104 cells were transfected with different rotavirus Wa expression plasmids in combination with rotavirus SA11 transcripts in order to examine the effect on cell viability (***Table 5.4***). Generally, it was observed that when rotavirus transcripts are transfected first, mass cell death occurred within 24 hours after the first transfection. Interestingly, when the plasmids encoding for all rotavirus proteins were transfected first, mass cell death was delayed approximately 24 hours after a transcript transfection. Furthermore, transfecting the plasmid combination encoding the replication core (VP1, VP2, VP3 and VP6), NSP1 or viroplasm-like structures (NSP2 and NSP5) first, also delayed the cell death caused by rotavirus transcripts. Transfecting plasmids encoding for either VP1 or VP6 had no delaying effect on the cell death caused by transfected transcripts.

MA104 cells were also transfected with different rotavirus SA11 expression plasmids in combination with rotavirus SA11 transcripts in order to examine the effect on cell viability (***Table 5.5***). Again it was observed that transfecting all SA11 encoding plasmids (Alpha-Delta) first, mass cell death caused by the second transcript transfection was delayed

approximately 24 hours. Transfecting the Beta (VP2 and VP3) and Gamma (VP4, NSP1 and VP6) first also resulted in postponing the cell death caused by transcripts. The Alpha (VP1, NSP2 and NSP5/6) and Delta (NSP3, VP7 and NSP4) did not influence the cell death caused by the second transfection of transcripts.

Finally, MA104 cells were transfected with the codon-optimised rotavirus SA11 plasmids in combination with rotavirus SA11 transcripts (***Table 5.6***). As observed with the rotavirus Wa encoding plasmids, transfecting plasmids encoding for the core replication complex first, delayed cell death by almost 24 hours.

None of the co-expression systems were able to deliver any viable rotaviruses after 3 blind passages. Although empty virus particles have been rescued from infected cells (Ayala-Breton et al. 2009), no evidence exists that empty virus particles are forerunners for infectious virus particles. Furthermore, in the core-filling model VP1 has no part in transporting ssRNA into the core particle, but studies with RNA interference have shown that the suppression of VP1 expression leads to accumulation of empty viral particles in infected cells (Ayala-Breton et al., 2009a). No experimental proof exists proposing that empty particles are able to be packaged with plus-sense ssRNA *in vitro*, nor have viral particles been rescued containing partially packaged dsRNA (McDonald and Patton, 2011). Moreover, taking into account the failure of the hybrid system to generate viable viruses, the core-filling model seems to be an unlikely mechanism for rotavirus RNA assortment and packaging.

5.4 Summary and possible future directions

In this chapter, the expression optimisation of three rotavirus plasmid-based sets was performed. Development of transcript-based reverse genetics systems for two other members of the *Reoviridae* family, bluetongue virus and African horsesickness virus, has been reported (Boyce et al., 2008, Matsuo et al., 2010). This study attempted to extrapolate the methods used these transcript-based reverse genetics systems to determine if *in vitro* derived rotavirus SA11 DLPs could be used to recover viable rotavirus. Unfortunately no viable rotavirus could be rescued. The development of a rotavirus transcript-only based reverse genetics system seems to be complicated due to the serious lack of fundamental

understanding about how the rotavirus genome is replicated and packaged. In contrast to a plasmid-based system, transfecting equimolar amounts of each genome segment's transcript are very difficult in a transcript-based system. The amount of transcripts generated during *in vitro* transcription is likely size-dependent, as it is during a normal infection (Ayala-Breton et al., 2009, Skehel and Joklik, 1969, Stacy-Phipps and Patton, 1987). Thus, the smaller transcripts will always be in abundance and simulating normal physiological conditions during an infection may be very challenging with a transcript only based system. For the successful development of a rotavirus transcript-based reverse genetics system in the future, special attention should be paid to the following elements: A systematic approach to evaluate every step of the transcript-based reverse genetics system is recommended. (1) Transfected rotavirus transcripts can be translated in a suitable *in vitro* cellular system. We showed the transcript derived expression of four different rotavirus proteins (VP1, VP6, NSP2 and NSP5). Ideally, the expression of all 12 rotavirus proteins should be confirmed. The lack of expression of only a single genome segment may have a grave effect on the viral replication cycle. (2) After expression of all viral proteins have been confirmed, the generation of an ssRNA (+) template for dsRNA synthesis and the efficacious packaging of the dsRNA genome into a viral particle should be investigated. In the current study, when transfecting DLPs, derived from the same pool as DLPs used for the *in vitro* transcription of transcripts, viable rotavirus could be recovered after the second blind passage. (3) A pre-existing DLP replication complex may be essential to successful replication and should be investigated further. (4) Lastly, the influence of the innate response to rotavirus transcripts is not completely understood and should be examined more closely.

In an effort to combine the transcript- and plasmid systems, a codon-optimised plasmid set was designed. A dual transfection strategy was followed. The codon-optimised plasmids encoded for the DLP-complex (VP1, VP2, VP3 and VP6), the viroplasm-like structure (NSP2 and NSP5) and the E3 ubiquitin ligase (NSP1). A second transfection of rotavirus SA11 *in vitro* derived transcripts were performed 24 hours after the first transfection. Transfecting codon-optimised plasmids first, 12 hours before transfecting rotavirus mRNA transcripts, noticeably delayed the mass cell death observed when only transfecting rotavirus transcripts. None of the hybrid systems were able to deliver a viable rotavirus. An expressed

replication core scaffold may not be able to recruit/import rotavirus mRNA as proposed by the core-filling model and it is recommended that this observation should be investigated further. Alas, after numerous attempts, no rotavirus could be recovered from either the transcript-only based- or plasmid/transcript reverse genetic systems.

Several key aspects of the rotavirus replication cycle are not well understood, making trouble shooting a defective rotavirus reverse genetic system a daunting task. For example, the detailed mandate of intracellular events controlling rotavirus genome replication and packaging are currently not known (Jayaram et al., 2004, McDonald and Patton, 2011, Silvestri et al., 2004, Trask et al., 2013). The formation of inclusion bodies (viroplasm-like structures) by NSP2 and NSP5 in uninfected cells has been shown (Fabbretti et al., 1999) but the exact mechanism of how the transcripts are localise in the viroplasm or the mechanisms that direct the synthesis of negative sense ssRNA, assortment and packaging of dsRNA segments into viral cores are still topics of heated debates (Desselberger et al., 2009, Richards et al., 2013, Silvestri et al., 2004).

The expression of rotavirus VP6 could be detected in all cell cultures (MA104, HEK 293H, BSR and COS-7) using immunostaining. In addition, the expression of transcript derived rotavirus VP1, NSP2 and NSP5 could be confirmed with immunofluorescence in MA104 and HEK 293H cells. This evidence is in contrast to a recent report which concluded that rotavirus ssRNAs are not translated in cells (Richards et al., 2013). The experimental approches of these two groups seem similar and unfortunatly it is not clear why DLP derived rotavirus ssRNAs are successfully translated in this system. A peculiar cell death pattern was observed in response to transfection of rotavirus transcripts which has been described before (Mlera, 2013, Uzri and Greenberg, 2013). Mass cell death was usually observed within 24 hours after exposure to rotavirus transcripts. However, the cell death did not seem to be due to normal viral CPE as no viable rotavirus could be recovered.

It was also observed that the transfection of certain plasmids encoding individual rotavirus Wa proteins (VP3, NSP1, NSP3, NSP4 and NSP5/6) led to mass cell death. Additionally, transfecting certain rotavirus protein encoding plasmid combinations, 12 hours before

transfecting rotavirus mRNA transcripts, noticeably reduced and delayed cell death. This delay in apoptosis/necrosis is intriguing and has not been observed before. The following chapter will investigate this in more detail and conclude that the effect is possibly due to the suppression of mechanisms of the innate immune response.