McDonald Lab
Rotavirus and the Fundamentals of Virology

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Contents

1 Lab Protocols

1.1 Location of Common Materials

Item	Location	Storage
(In)complete Medium 199	Incomplete Medium 199 is located in the cold room in the hallway immediately outside the lab.	Complete and serum-free Medium 199s are stored in the 4°C freezer in R2048.
Penicillin/Streptomycin Stock	P/S stock is located in the −20°C freezer in the hallway immediately outside the lab.	Leftover stock is stored in the 4°C freezer in R2048.
L-Glutamine Stock	Stock is located in the −20°C freezer in the hallway immediately outside the lab.	Leftover stock is stored in the 4°C freezer in R2048.
Amphotericin B stock	Stock is located in the −20°C freezer in the hallway immediately outside the lab.	Leftover stock is stored in the 4°C freezer in R2048.
0.05% Trypsin-EDTA	Stock is located in the −20°C freezer in the hallway immediately outside the lab.	Leftover stock is stored in the 4°C freezer in R2048.
Trypsin (2mg/mL)	Stock is located in the −20° freezer in the hallway immediately outside the lab.	Leftover stock should be refrozen in a box labelled with your name.
Fetal Bovine Serum	Stock is located in the −20°C freezer in the hallway immediately outside the lab.	Leftover stock is stored in the 4°C freezer in R2048.
(In)complete 2x EMEM	Stock is located in the cold room in the hallway immediately outside the lab.	Complete EMEM stock is stored in the 4°C freezer in R2048.
SeaPlaque Agarose	Stock is located in a jar above the benches immediately outside of the biosafety cabinet.	Leftover stock should be replaced where you found it.
10x PBS	Stock is located in a plastic bottle above the benches immediately outside of the biosafety cabinet.	Leftover stock should be replaced where you found it.
SA11 Stock	Stock is located in the −20° freezer in the hallway immediately outside the lab.	Leftover stock should be replaced where you found it.
Natural Red	Stock is located in the 4° freezer in R2048.	Leftover stock should be replaced where you found it.

1.2 Recording Work and Labelling Materials

All solutions should be labeled with your name, the date of preparation, and what the solution contains. All cell flasks should be labeled with your name, the date of preparation, the type of cell contained, and the passage of cell contained.

All work completed in the lab should be recorded in a laboratory notebook. Each page should

contain entries from only a single day. The date of entry should be recorded at the top of each page. All writing must be easily readable and written in pen. Any errors should be struck through with a single solid line with the correction appearing next to it. Any space on a page not used at the end of a work day should be clearly crossed out in pen.

1.3 Procedures for Autoclaving

Ensure that all items have autoclave tape (if necessary). Place all items into the plastic bin found on the cart next to R2048. Take to the autoclave room. Add an autoclave quality indicator strip to the bin and insert into the autoclave. Close the door and select the appropriate options based on what is being sterilized. Start the cycle and fill out the autoclave use form on the bench next to the machine. Once the cycle is complete, retrieve the bin using the thick insulated gloves found in the lab (next to where the bin is stored). It is normal for there to be a small amount of water in the base of the bin.

1.4 Preparation of Medium 199 (Serum-Free)

Items Needed:[1]

1. Incomplete Medium 199 (500mL)
2. Penicillin/streptomycin stock (5mL)
3. Amphotericin B stock (1mL; 250μg/mL)

In the biosafety cabinet, supplement 500mL incomplete Medium 199 with 5mL P/S stock and 1mL amphotericin B stock. Store at 4°C for up to 3 months.

1.5 Preparation of Medium 199 (Complete)

Items Needed:[2]

1. Incomplete Medium 199 (500mL)
2. Penicillin/streptomycin stock (5mL)
3. Amphotericin B stock (1mL; 250μg/mL)
4. Fetal bovine serum (55mL)

In the biosafety cabinet, supplement 500mL incomplete Medium 199 with 5mL P/S stock, 1mL amphotericin B stock, and 55mL fetal bovine serum. Store at 4°C for up to 3 months.

1.6 Preparation of 1.2% Agarose

Items Needed:

1. SeaPlaque agarose
2. Milli-Q filtered water

To a 500mL flask add 1.2g agarose for every 100mL water. Inadvisable to fill flask to more than 400mL. Cap and shake. Loosen lid. Apply autoclave tape to the lid. Autoclave approx. 20 min.

[1]The paper *Culturing, Storage, and Quantification of Rotaviruses* advises using different quantities of some materials below. Nonetheless, the following are the recommended quantities for use in lab.

[2]See Note 1.

1.7 Preparation of 2x EMEM (Serum-Free)

Items Needed:

1. incomplete 2x EMEM (500mL)
2. 200mM L-glutamine (10mL)
3. P/S stock (10mL)
4. 250μg/ml amphotericin B stock (1mL)

In the biosafety cabinet, to 500mL incomplete 2x EMEM stock add 10mL L-glutamine, 10mL P/S stock, and 1mL amphotericin B stock. Store at 4°C for up to 3 months.

1.8 Preparation of PBS (Phosphate Buffered Saline)

Items Needed:

1. 80mL 10x PBS
2. 720mL milli-Q water

To a large graduated cylinder add 80mL 10x PBS solution (use a 25mL pipet). Fill the graduated cylinder to 800mL with milli-Q-filtered water. Apply autoclave tape to the lid. Autoclave for 30 minutes.

1.9 Procedure for Splitting MA104 Cells

Items Needed:

1. 1x PBS
2. 0.05% Trypsin-EDTA
3. Complete Medium 199
4. 150cm^2 flask

In a water bath, warm PBS, Trypsin, and complete Medium 199 to 37°C. Transfer all materials into the biosafety cabinet. Tilt the flask with your cells (having formed a confluent monolayer) such that the culture medium runs towards the neck of the flask. Vacuum out all culture medium using a small glass pipet inserted into the rubber vacuum hose. Dispose of the pipet. To the cell culture, add 10mL 1x PBS. Tilt the flask to ensure that all cells are thoroughly covered by PBS. Aspirate using a small glass pipet. Dispose of the pipet.

To the cell culture, add 5mL Trypsin, tilting the flask forward and back to ensure that the cells are fully bathed. Vacuum out the Trypsin with a small glass pipet. Add a second 5mL portion of Trypsin to the cell culture. Incubate at 37°C until all cells have detached from the surface and are free-floating. Lightly tap the flask with your hand if any cells remain attached.

To the cell culture add 15mL complete Medium 199. (If you used less trypsin in the previous step, adjust the amount of Medium 199 applied such that there is 20mL solution in the flask.)

To each new flask that you wish to prepare, add complete Medium 199 such that the final flask volume after the cell mixture is added will be equal to 25mL. To determine the volume of cell mixture to add to each new flask, we use Table 1.1.

Cap the new flask(s) and tilt forward and back to evenly spread the cells. Loosen the lids and incubate at 37°C.

Table 1.1

Cell dilution ratio	Cell mix volume to add
1:2	10mL
1:4	5mL
1:8	2.5mL

Note. For example, if we wished to prepare one 1:4 dilution and two 1:8 dilutions, to our first flask we would add 15mL complete Medium 199 and 10mL cell mixture; to each of our second two flasks we would add 20mL complete Medium 199 and 5mL cell mixture.

1.10 Procedure for Plating MA104 Cells

Items Needed:

1. 1x PBS
2. 0.05% Trypsin
3. Complete Medium 199
4. Trypan Blue
5. 6-well plates (x4)

Aspirate the cell culture medium from the flask. Wash the cell monolayer in 10mL $1x$ PBS and aspirate from the flask. Wash the cell monolayer in 5mL trypsin and aspirate from the flask. Bathe the cell monolayer in 5mL trypsin and incubate at 37°C until all cells have detached from the flask. (Check every 2 minutes.) Tap the flask with your hand to detach any remaining cells.

To the flask add 15mL complete Medium 199 and mix thoroughly with the pipet. On a piece of parafilm, combine 10μL cell mixture and 10μL trypan blue. Mix thoroughly with a pipet. To a slide, add 20μL such that the slide is completely filled with liquid. Insert into the automatic cell counter and record the result (total cells; live cells; percent alive). Use this to extrapolate the number of cells in the T150 flask and in each well.

To a 100mL conical vial add 65mL complete Medium 199. Supplement this with 10mL cell solution. (These values may be doubled if 8 plates are being prepared. A T75 flask can accommodate 4 6-well plates; a T150 flask 8 6-well plates.) Transfer the fell mix to each of the wells, adding 3mL cell solution per well. Spread the cells by tilting forward and back. Incubate the plates at 37°C for several days.

1.11 Procedure for activating RV SA11 and Infecting MA104 Cells

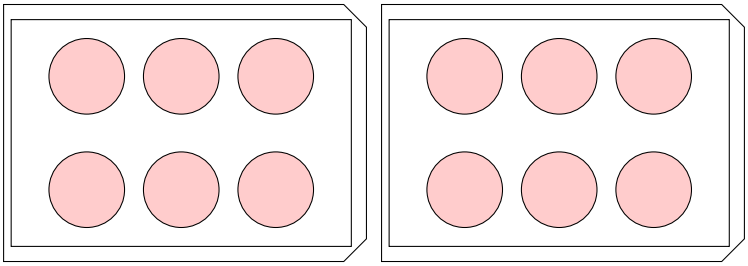
Items Needed:

1. SA11 stock
2. Trypsin (2mg/mL)
3. Serum-free Medium 199
4. 2x EMEM
5. 1.2% agarose

In a large Eppie tube combine 400μL rotavirus SA11 stock and 2μL trypsin. Cap and incubate in a 37°C water bath for 1 hour. To each of 8 15mL plastic tubes add 2.7mL serum-free Medium 199.

Add 300μL virus mix to the first tube and label as 10^{-1}. Mix the solution by vertex to homogenize. Take 300μL of the 10^{-1} solution and add to the second tube. Label 10^{-2}, cap, and mix by vertex. Repeat this process across the remaining tubes, ending with a viral concentration of 10^{-8}. Store in the 4°C freezer if the cells are not going to be immediately infected. This is enough for 2 6-well plates.

To 2 6-well plates, add the rotavirus titers. Each titer is to be performed in duplicate. To achieve this, add $\sim$ 1mL of the 10^{-8} titer to two wells, then 10^{-7} titer to two wells, and so on. You will end with two plates as follow:



Incubate the plates at 37°C for 1 hour.

Liquify a jar of 1.2% agarose using a microwave. Warm 2x EMEM to 37°C in a water bath. For each plate infected, prepare a solution of 10mL agarose, 10mL 2x EMEM, and 5μL trypsin. (Add the EMEM first, followed by the trypsin, followed by the agarose. Do not add trypsin to hot agarose.)

One plate at a time, aspirate the viral inoculant. (You may use a new pipet for each well or may use one pipet per plate, aspirating from the lowest concentration well to highest.) Quickly add to each well $\sim$ 3mL of the agarose solution. Place the lid on the well and allow it to sit, undisturbed, for approximately 30 minutes (or until the agarose overlay has solidified). Incubate for several days at 37°C.

1.12 Procedure for Performing RV Plaque Assay

1.13 Procedure for Transducing MA104 Cells with siRNA-Expressing Lentiviral Vectors

2 Rotavirus

2.1 History

Human rotavirus (RV) was discovered in 1973 by Bishop et al. utilizing direct visualization by electron microscopy. Approximately concurrently, simian, murine, O, and bovine agents were discovered that later would be additionally classified as rotaviral.

2.2 Classification

RVs comprise the genus *Rotavirus* within the family *Reoviridae*. Some features are that:

1. the mature viruses are about 100nm ($1,000$Å) in diameter with a triple-layered icosahedral protein capsid being comprised of outer and intermediate layers and an inner core;
2. 60 protein spikes protrude from the outer shell;
3. calcium is required to maintain the integrity of the outer shell;
4. particles contain an RNA-dependent RNA polymerase and related enzymes able to produce capped RNA transcripts;
5. the virus genome contains 11 dsRNA segments;
6. genetic reassortment can occur between two rotaviruses of the same group; and
7. the virus particles, uniquely, are formed by migration into the ER where enveloped particles are formed.

RVs are classified into serogroups of multiple serotypes each. An RV group includes viruses that share cross-reacting antigens. There are 7 distinct groups that rotaviruses compose. Group A, B, and C RVs are found in both humans and animals; group D, E, F, and G RVs have only been observed in animals to date.

Group A RVs have been predominantly identified as causing diarrheal disease in infants and mammalian and avian young. Group B RVs are associated with severe diarrheal epidemics. Group C RVs have been occasionally reported in family outbreaks.

Table 2.1

Genome Segment	Protein Product	Location	N/virion	Ts mutant group	Function
1	VP1	Core	12	C	RNA-dependent RNA polymerase, ss-RNA binding, complex with VP3
2	VP2	Core	120	F	RNA binding, required for replicase activity of VP1
3	VP3	Core	12	B	Guanylytransferase, methytransferase, ss-RNA binding, complex with VP1
4	VP4	Outer capsid	120	A	Hemagglutinin, cell attachment, neutralization antigen, protease enhanced infectivity, virulence, putative fusion region
5	NSP1	Nonstructural		NA	Basic, zinc finger, RNA binding, virulence in mice; interacts with and degrades IRF-3; nonessential for some strains
6	VP6	Inner capsid	780	G	Hydrophobic, trimer, subgroup antigen, protection; required for transcription
7	NSP3	Nonstructural		NA	Acidic dimer, binds 3′ end of viral mRNAs, competes with cellular PABP for interaction with elF-4G1, inhibits host translation
8	NSP2	Nonstructural		E	Basic, RNA binding, oligomer, NTPase, helicase, forms viroplasms with NSP5
9	VP7	Outer capsid	780	NA	RER integral membrance glycoprotein, calcium-dependent trimer, neutralization antigen
10	NSP4	Nonstructural		NA	RER transmembrance glycoprotein, intracellular receptor for DLPs, role in morphogenesis, interacts with viroplasms, modulates intracellular calcium and RNA replication, enterotoxin, secreted cleavage product, protection by antibody, virulence
11	NSP5	Nonstructural		NA	Basic phosphoprotein, RNA binding, protein kinase, forms viroplasms with NSP2, interacts with VP2 and NSP6
	NSP6	Nonstructural		NA	Interacts with NSP5, present in viroplasms and most virus strains

Note. Table of rotavirus proteins and their relevant data. Adapted from Fields Biology, 5e.

2.3 Virion Structure

Three-dimensional reconstructions using cryo-electron microscopy have revealed that RV particles possess icosahedral symmetry with a $T = 13l$ icosahedral surface lattice for the two outer layers. There exist also 132 large channels $\sim 140\mathring{A}$ deep that span both shells and link the outer surface with the inner core. The virion is schematically represented in Figure 1).

Three types of channels have been identified based on position and size. Specifically, there are 12 type I channels running down the icosahedral fivefold axes; 60 type II channels around the fivefold axes; and 60 type III channels around the threefold icosahedral axes.

Further, 60 spikes extend from the surface of he outer shell. These protein spikes lit on the edges of the type II channels and are composed of VP4. The spikes have a distinct structure with two distal globular head domains, a central body, and an internal globular domain tucked inside the VP7 layer in the type II channels.

2.4 Genome Structure

The viral genome of 11 dsRNA segments is contained within the core capsid. The virus particles have been shown to contain their own RNA-dependent RNA polymerase to transcribe the individual RNA segments into active mRNA. (I.e., deproteinized RV dsRNA are non-infectious.)

Each positive-sense RNA segment starts with a $5'$-guanidine followed by a set of conserved sequences that are part of the $5'$-noncoding sequences. An open reading frame (ORF) coding for the protein product and ending with the stop codon follows, and then another set of noncoding sequences is found containing a subset of conserved terminal $3'$-terminal cytidines.

One of the most intriguing aspects of RV replication relates to the mechanism(s) of how it coordinately replicates and packages the 11 viral mRNAs. These 11 mRNAs must share common cis-acting signals because they are all replicated by the same polymerase, and the UGUG sequence of the consensus sequence is reorganized in a base-specific manner by the polymerase. Additionally, each mRNA must contain a signal that is unique to it alone because the 11 mRNA must be distinguished from one another during packaging. Generally, the conserved terminal sequences in genome segments contain cis-acting signals that are important for the transcription, RNA translation, RNA transport, replication, assembly, or encapsidation of the viral genome segments. Some of the cis-acting signals for RV RNA replication and translation have been identified (Figure 2), but assembly or encapsidation signals remain unknown. The highly conserved noncoding regions of the RNA may contain the gene-specific packaging signals.

2.5 Coding Assignments

The coding assignments and many properties of the proteins encoded in each of the 11 genome segments are not well established (cf. Table 2.1) although new protein functions continue to be identified. The rotavirus genome segments code for structural proteins found in virus particles and nonstructural proteins found in infected cells but not present in mature particles. Six of the genome segments code for structural proteins found in virus particles. Another 6 proteins are nonstructural.

The nomenclature of the viral proteins designates structural proteins as viral protein (VP) followed by a number with VP1 being the highest molecular-weight protein. Nonstructural proteins are designated as nonstructural protein (NSP) followed again by a number.

2.6 Stages of Replication

The general features of rotavirus replication (detailed in Figure 3) are as follows:

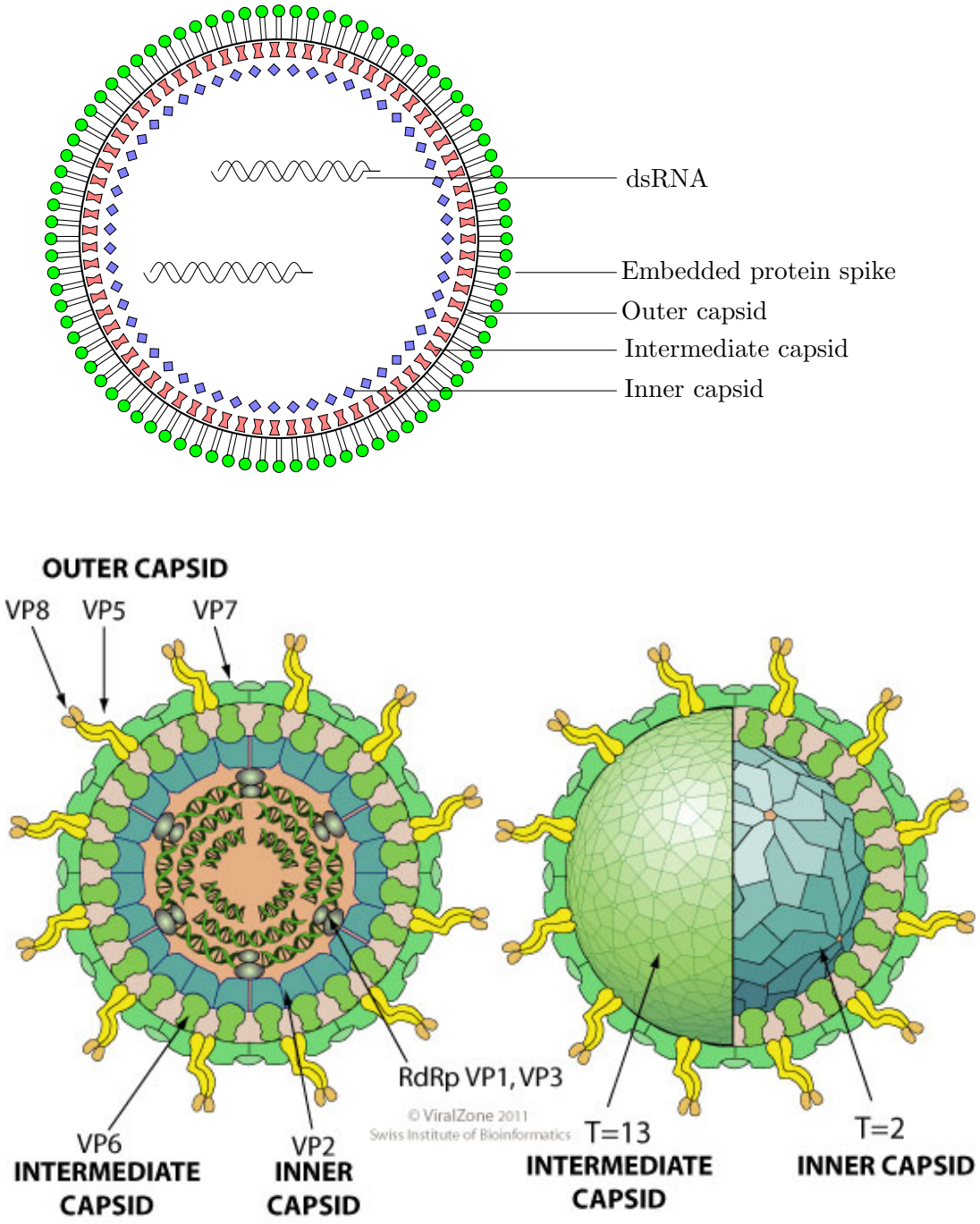


Figure 1: Schematic illustration of a rotavirus particle, laterally bisected (top) accompanied by an alternate representation showing the specific structural proteins (bottom left). The structures of the virion's three capsids is also represented (bottom right).

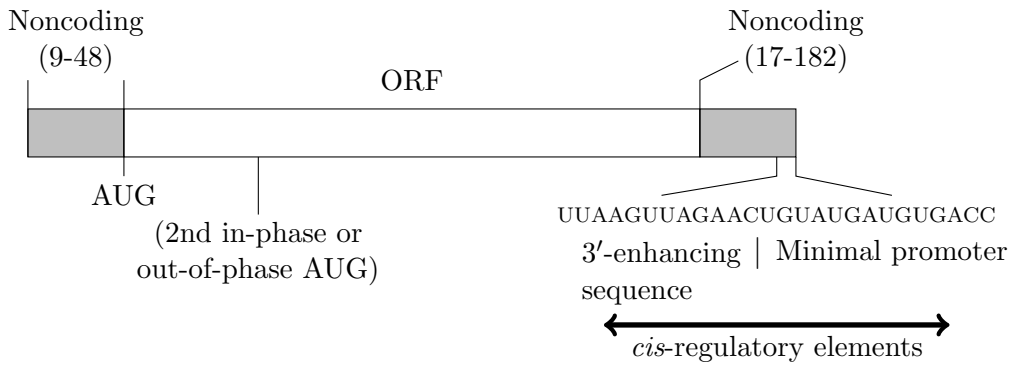


Figure 2: Major features of rotavirus gene structure. Schematic shows the overall structure of RV genes derived from published sequences of genes 1 through 11. All 11 RV genes lack a polyadenylation signal, are A+U rich, and contain conserved consensus sequences at their 5′ and 3′ ends.

1. Cultivation of most virus strains requires the addition of exogenous proteases to the culture medium. This ensures activation of viral infectivity by cleaving the outer capsid protein VP4.
2. Replication is totally cytoplasmic.
3. Cells do not contain enzymes to replicate dsRNA; the virus must supply the necessary enzymes.
4. Transcripts function both to produce proteins as a template for production of negative strand RNA. Once the complementary negative strand is synthesized, it remains associated with the positive strand.
5. The dsRNA segments are formed within nascent subviral particles and free dsRNA or free negative-stranded ssRNA is never found in infected cells.
6. RNA replication occurs within cytoplasmic viroplasms.
7. Subviral particles form in association with viroplasms and these particles mature by budding through the membrane of the ER. In this process particles acquire their outer capsid proteins.
8. Levels of intracellular calcium are important for controlling virus assembly and integrity.
9. Cell lysis releases particles from infected cells grown on monolayers.

2.6.1 Attachment

The molecular details of rotavirus adsorption, entry and uncoating are complex and remain incompletely understood. As expected from their locations in the virus structure, VP4 and VP7 are implicated in the initial interactions with host cells. Given that a broad range of cells can bind rotaviruses and be infected with different efficiencies, suggesting that initial attachment is a promiscuous interaction with postattachment receptors being critical for virus entry into the cell.

2.6.2 Penetration and Uncoating

It seems likely that RV entry into the cell is a coordinated, multistep process involving sequential interactions with several ligand and that involves a series of conformational changes in the capsid proteins. The

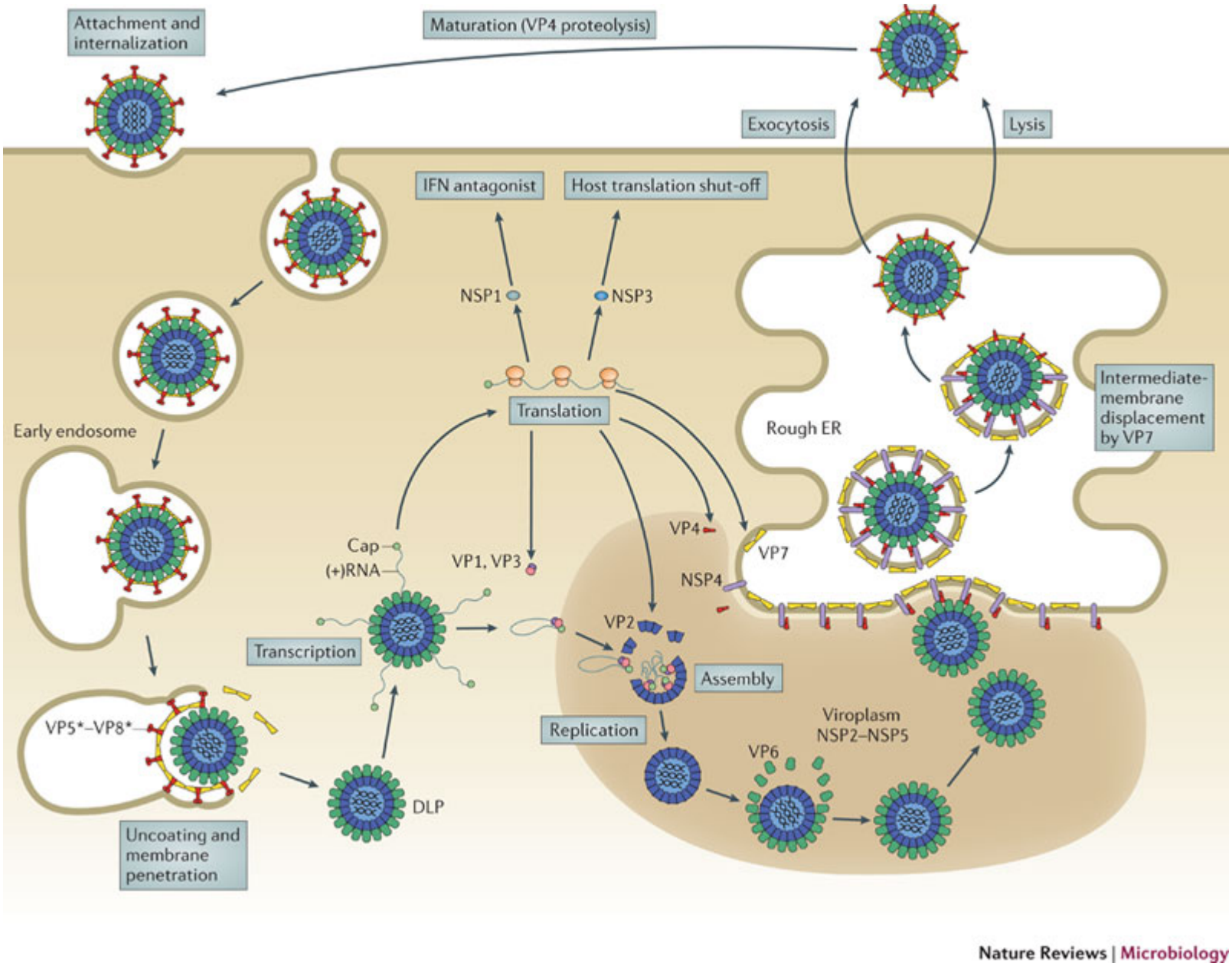


Figure 3: Stylized representation of rotavirus replication.