The role of NSP1 in the regulation of rotavirus gene expression
by Dana Nicole Mitzel

A thesis submitted in partial fulfillment of the requirements for the degree. of Doctor of Philosophy in Veterinary Molecular Biology
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Abstract:
During rotavirus replication, there is transcriptional and translational control of rotavirus gene expression; however, the molecular mechanisms that regulate expression of each the rotavirus genes are not well defined. Therefore, we are investigating the mechanisms of regulation of rotavirus gene expression and the possible involvement of nonstructural protein NSP1. The identification of a potential mechanism for the regulation of rotavirus gene expression was evaluated using two viral genes that are expressed at different levels early in the infection. VP6, encoded by gene segment 6, is expressed in excess over NSP1, which is encoded by gene segment 5. The mRNA levels of the two genes were measured and no significant difference was found. The half-life of the two proteins was calculated to be the same, indicating that the stability of NSPl and VP6 are similar. Polysome analyses demonstrated that gene 6 mRNA is translated more efficiently than gene 5 mRNA, and further studies of gene 5 illustrated that gene 5 mRNA is a poor template for initiating translation. Therefore, one mechanism responsible for the difference in the levels of expression of gene 5 mRNA and gene 6 mRNA occurs at the level of translation initiation.

To investigate the role of NSP1 in regulating viral gene expression, the sedimentation of rotavirus mRNAs in polyribosome gradients were compared between a mutant viral strain lacking NSP1 and a wildtype strain. Gene 6 mRNA showed no difference in sedimentation at four and six hours post infection. However, when the sedimentation of gene 6 at two hours post infection was examined, a greater percentage of gene 6 mRNA of the mutant strain sedimented in the polysomal fractions compared to wildtype gene 6 mRNA. Gene 11 mRNA showed little difference in the sedimentation at two hours post infection, but at 4 and 6 hours post-infection, a greater percentage of gene 11 mRNA sedimented in the polysome fractions for the mutant strain compared to the wildtype strain. These data suggest that rotavirus genes are differentially regulated and that NSP1 may participate in the regulation of viral gene expression.

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APPROVAL

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Dana Nicole Mitzel

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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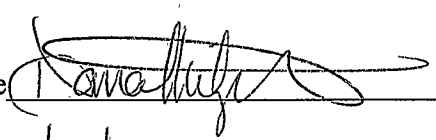
Signature 

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ABSTRACT

During rotavirus replication, there is transcriptional and translational control of rotavirus gene expression; however, the molecular mechanisms that regulate expression of each the rotavirus genes are not well defined. Therefore, we are investigating the mechanisms of regulation of rotavirus gene expression and the possible involvement of nonstructural protein NSP1. The identification of a potential mechanism for the regulation of rotavirus gene expression was evaluated using two viral genes that are expressed at different levels early in the infection. VP6, encoded by gene segment 6, is expressed in excess over NSP1, which is encoded by gene segment 5. The mRNA levels of the two genes were measured and no significant difference was found. The half-life of the two proteins was calculated to be the same, indicating that the stability of NSP1 and VP6 are similar. Polysome analyses demonstrated that gene 6 mRNA is translated more efficiently than gene 5 mRNA, and further studies of gene 5 illustrated that gene 5 mRNA is a poor template for initiating translation. Therefore, one mechanism responsible for the difference in the levels of expression of gene 5 mRNA and gene 6 mRNA occurs at the level of translation initiation.

To investigate the role of NSP1 in regulating viral gene expression, the sedimentation of rotavirus mRNAs in polyribosome gradients were compared between a mutant viral strain lacking NSP1 and a wildtype strain. Gene 6 mRNA showed no difference in sedimentation at four and six hours post infection. However, when the sedimentation of gene 6 at two hours post infection was examined, a greater percentage of gene 6 mRNA of the mutant strain sedimented in the polysomal fractions compared to wildtype gene 6 mRNA. Gene 11 mRNA showed little difference in the sedimentation at two hours post infection, but at 4 and 6 hours post-infection, a greater percentage of gene 11 mRNA sedimented in the polysome fractions for the mutant strain compared to the wildtype strain. These data suggest that rotavirus genes are differentially regulated and that NSP1 may participate in the regulation of viral gene expression.

CHAPTER ONE

ROTAVIRUSES AND REGULATION OF GENE EXPRESSION

Rotaviruses

Introduction

Rotavirus, a member of the *Reoviridae* family, derived its name from the Latin word *rota,* meaning wheel [1]. This name was suggested due to the appearance of the virions in electron micrographs. The complete virions appeared to have a wide hub with short spokes and a thin circular rim [1]. During the 1930's and 1940's in the United States, the disease was first reported in suckling mice as epizootic diarrhea of infant mice (EDIM), but was never characterized [2-5]. EDIM was finally characterized as a virus (rotavirus) different from other mouse viruses known at that time [6-8]. In 1969, a virus described as reovirus-like was isolated from cattle and was the first rotavirus to be adapted for continuous subculture in cells. It was subsequently characterized and confirmed as the cause of diarrhea in calves [9]. It was not until the mid 1970's that rotavirus was identified in young children via electron microscopy [10]. Virus particles morphologically indistinguishable from the viruses described in mice, calves, and children, were also found in the feces of other animals such as pigs, deer, and rabbits [11-18]. Today, rotaviruses are recognized as the chief etiologic agent of viral gastroenteritis in the young of most avian and mammalian species [19-24].

Rotaviruses are classified into seven distinct serogroups (A-G) [25]. Group A rotaviruses are the most common serogroup found in the young of both humans and animals. Little is known about groups B-G, since non-group A rotaviruses are difficult to cultivate and until recently no diagnostic tests for the non-group A rotaviruses were available [26, 27]. The serogroups are determined by viral protein (VP) 6, which is the most abundant rotavirus protein [28, 29]. Different viral strains within the same serogroup share common antigens that reside in VP6, and these antigens can be detected by monoclonal antibodies to determine the serogroup of the strain [30-32]. Group A rotaviruses are further categorized into serotypes. The two viral surface proteins, VP4 and VP7, each evoke an antibody response that neutralizes virus infectivity in vitro and are therefore used to determine the serotypes [28, 33]. Because the genes that encode VP4 and VP7 can reassort independently of each other and any virus isolate can possess heterologous neutralization antigens, a binary classification system of G and P serotypes was proposed [28]. The P serotype denotes the protease sensitive outer capsid protein VP4 and G denotes the glycoprotein VP7 [28]. Thus far, 15 G serotypes and at least 21 P types have been identified [34]. Various combinations of P and G serotypes can be found, creating great diversity in the antigenic presentation of the virus [35].

Development of vaccines is complicated by this antigenic diversity, as most data indicate that protection from rotavirus is primarily homotypic, and protection against heterotypic infections is more difficult to achieve [36, 37]. Therefore, research efforts have focused on understanding the biology of rotavirus infections from both a

molecular and immunological, or host response standpoint. A summary of rotavirus disease mechanisms and mechanisms of virus replication is presented in the following sections.

Rotavirus Gastroenteritis

Rotavirus infects the mature enterocytes in the mid and upper villous epithelium in the small intestine [38-40]. The infection leads to cell death and shedding of the infected cells causing the villi to become stunted and shortened [38, 41, 42]. In order to repopulate the epithelium, cells migrate from the crypt to the mucosa without becoming fully differentiated, thereby replacing the absorptive villous epithelium with the immature secretory crypt cells. Resulting crypt cell hyperplasia is accompanied by hypersecretion and malabsorption, which contributes to the diarrhea [43-45]. However, this malabsorption is not the entire story to rotavirus pathogenesis because it fails to explain the occurrence of diarrhea prior to villus blunting [45]. Other mechanisms proposed to explain rotavirus-induced diarrhea include activation of the enteric nervous system [43] and intestinal secretion stimulated by rotavirus nonstructural protein NSP4 [46]. NSP4 was identified as the first viral enterotoxin [46]. Enterotoxins stimulate net secretion in intestinal segments in the absence of histological changes [47]. Cleavage products of NSP4 are found in the medium of infected cells and this product retains the enterotoxin activity [48]. Binding assays demonstrated that cells possess an unknown receptor to NSP4 [47]. When human intestinal cells are exposed to exogenously added NSP4, NSP4 initiates mobilization of calcium (Ca^{2+}) [49]. This increase in intracellular Ca^{2+} causes the release of chloride (Cl^-) through a Ca^{2+} dependent pathway and induces

diarrhea in mice [46, 50]. Antibodies to NSP4 can passively protect against rotavirus disease in the neonatal mouse model, further suggesting a role for this protein in rotavirus pathogenesis [47].

Rotavirus also activates the enteric nervous system. The activated nerves stimulate cells of the intestinal lining to increase water secretion, resulting in diarrhea [43]. How rotavirus activates the enteric nervous system is not known, but several mechanisms have been proposed. First, activation of the enteric nervous system could be due to rotavirus infecting neurons [43, 51]. Alternatively, the enterotoxic effects of NSP4 could possibly activate the enteric nervous system. Finally, the increase in intracellular Ca^{2+} by NSP4 could trigger the release of amines or peptides from endocrine cells of the gut and stimulate dendrites or free nerve endings underneath the epithelial layer, thereby activating the secretion by the intestinal lining [43].

Several factors can influence the severity of the disease. Severe disease results when the young are exposed to high doses of virus or a highly virulent strain, which often occurs in a confined highly contaminated area [52-54]. The highly virulent strains replicate more quickly, which increases the rate of enterocyte death and shedding in the villous epithelium, thereby increasing the severity of the disease. The area of rotavirus infected epithelium can be up to eight times greater with the more virulent strain when compared to a less virulent strain [55]. Management and environmental factors, such as early weaning, also can affect the severity of the disease [56-59]. Passive immunity also is important especially in calves, which are born antibody deficient and acquire immunoglobulins via ingestion of colostrum [53]. Many rotavirus infections of

neonatal calves are mild or subclinical due to the maternal rotavirus antibodies secreted in the milk [53]. Weaning at an early age curtails the milk intake and decreases protection provided by the maternal antibodies [53, 57]. Similarly in humans, breastfeeding provides a partial protective effect [59].

Mixed infections with rotavirus and other enteropathogens can increase the severity of the disease [40, 60-63]. Studies have shown that rotavirus infections enhance the colonization of bacteria, such as *E. coli* and *Clostridium,* in the intestinal tract [61-63]. Simultaneous experimental infections of calves with *E. coli* and rotavirus cause diarrhea under circumstances in which neither pathogen alone would cause diarrhea [64]. Experiments in mice also show a synergistic effect. In mice, there is a greater mortality with mixed infections of rotavirus and *E. coli* than with either agent alone [62]. The mutual enhancement of pathogenecity in mixed infections occurs from the mechanisms by which the agents cause diarrhea. Due to the enterotoxigenic effect of both agents, there is an increase of fluid secretion in the gastrointestinal cells. Rotaviruses further facilitate diarrhea by restricting fluid absorption, because of absorptive cells destruction at the villous tips. [38, 41, 42, 44-46, 62].

Rotavirus disease also is age-dependent. Most adults are resistant to the disease, but not to infection [46, 65, 66]. The mechanisms for age-dependent resistance to disease are unknown, but could be due to several factors. One possibility is a difference in the receptors presented on the villus epithelial cells [46, 66, 67]. In mice, the peak age at which rotavirus binds to enterocytes is dependent upon the age of the mouse [66]. Further studies demonstrate that when enterocytes undergo premature

maturation by the addition of cortisone acetate, the mice are less susceptible to rotavirus-induced diarrhea [67]. Differences in gut biology also could play a role in age-dependent disease. In older animals, the rate of natural enterocyte replacement is faster than in the younger animals, and therefore, the older animals do not suffer from the malabsorption and increased fluid secretion as compared to the young [68, 69]. Finally, protection of rotavirus-induced diarrhea is dependent upon serotype specificities and the levels of neutralizing antibodies. Serotype specificity of the humoral immune response is dependent upon previous exposure, and therefore, over time adults are exposed to the most common serotypes and acquire some protection [70, 71]. Taken together, the data outlined above suggest that mechanisms of disease and disease resistance are multifactorial and not yet completely defined.

Rotavirus Epidemiology and Prevention in Children

Rotavirus infections are endemic and most prevalent during the winter months in temperate areas [72]. In children, rotavirus-induced disease is due to multiple serotypes present in a local reservoir within a community. Therefore, the serotypes present in one town may not be the same serotypes found in the neighboring towns [73]. It is estimated that children under the age of five in the United States have 1.3-2.3 episodes of gastroenteritis a year and this rate increases three-fold for children in daycare [74]. Rotavirus is the major player in these episodes, causing 30-60% of the cases [65, 72, 75, 76]. By the fifth year of age, it is estimated that almost every child will be affected by rotavirus-induced disease [77]. The peak incidence for rotavirus infection is between the ages of three and fifteen months, with children over three years

of age rarely symptomatic for severe disease [78]. One in five children affected by this disease require a clinic visit, while one in sixty-five need an inpatient stay. The incidence of deaths caused by rotavirus is on average 440,000 children under the age of five each year [77]. In the United states alone, the annual health care costs exceed one billion dollars [79]. This high occurrence of rotavirus in children worldwide underlines the need for vaccines.

In 1998, Rotashield®, a live attenuated tetravalent rotavirus vaccine was marketed for use in the United States [80]. A year later, Rotashield® was withdrawn from the market due to its association with intussusception in infants given the vaccine [81]. Rotashield® uses an antigenically related rhesus rotavirus strain as the immunogen to induce protection against the four most common rotavirus serotypes G1-4 [82, 83]. Different efficacy rates of the vaccine were observed during the prelicensure trials [30, 83]. In the United States and Finland, the efficacy rate of the RRV-TV was 61-100% for severe disease. An 88% efficacy rate was observed in Venezuela, while Brazil showed a 0-46% efficacy rate for severe disease. In Brazil, uncommon serotypes are found that represent one-third of the infections, demonstrating that future vaccines may require a better understanding of the molecular epidemiology of wildtype rotaviruses circulating in a given population and may need to include a broader spectrum of serotypes [75, 84]. As of 2001, several monovalent and quadrivalent vaccine candidates are undergoing various safety, immunogenecity, and efficacy trials [79].

Rotavirus Epidemiology and Prevention in Calves

It is estimated that in North America approximately 5% of calves less than one month old die from diarrhea. These losses cost the cattle industry between one and seven billion dollars annually [22, 23, 54]. In calves, the highest occurrence of rotavirus disease occurs between one to three weeks of age [24, 85, 86]. Most adult cows are seropositive to rotavirus, and therefore, transfer various degrees of passive immunity to nursing calves [20, 21, 57, 58, 87-89]. Many neonatal calves have mild or subclinical rotavirus infections, possibly due to passive immunity obtained from dams [20, 53, 90, 91].

Two vaccination approaches have been created to manage the disease in calves. Attenuated oral vaccines are used to stimulate the active immunity of the calves. Oral vaccination was successful in gnotobiotic calves and in some field studies [56, 92], but the efficacy of the vaccine was poor in other double blind studies [20, 54]. The low efficacy of the vaccine could be due to several factors. First, the virus strain used in the vaccine may not give heterotypic protection from an infection with a different serotype circulating in the population [53]. Second, a calf could be exposed to a virulent strain before protection from the vaccination is induced [54, 93]. The protective effect of the vaccine could be overwhelmed if the vaccinated calves are exposed to diarrheic calves, due to exposure to high doses of the virus [54]. DeLeeuw also proposed that the lack of protection induced by the vaccine is due to neutralization of the vaccine virus by antibodies present in the colostrum [93]. Since the calves can be exposed to the virus a

short time after birth, it becomes necessary to handle and vaccinate each calf shortly after birth. This creates management problems for large herds [53].

The inconsistencies found with the oral vaccine led to the use of passive immunization in cows. Pregnant dams are vaccinated with a live attenuated rotavirus strain to increase the titers of antibodies, thereby prolonging the secretion of the antibodies in the colostrum and milk [21, 57, 58, 94-96]. The efficacy of the commercial bovine rotavirus maternal vaccine varied in field trials [21, 58, 94, 97]. This could be due to several factors, such as the presence of other pathogens, the lack of adjuvants, and the low dose of rotavirus in the vaccine [56-58, 89, 96-101].

Rotavirus is the major cause of severe debilitating diarrhea in the young of most mammalian species. There is great diversity in the antigenic presentation of the virus among the circulating strains. This diversity makes it difficult to obtain protection against the large range of serotypes. Therefore, understanding mechanisms involved in the regulation of gene expression at the molecular level, can lead to new antiviral interventions that are independent of the antigenic type circulating in an infected population.

Rotavirus Structure and Genome Content

The rotavirus particle is of icosahedral symmetry [102, 103]. The mature virion is nonenveloped and consists of three concentric protein layers, as shown in Figure 1.1 [104-106]. The outer capsid consists of VP7 trimers and 60 spike-like VP4 dimers [105]. VP4 interacts with VP7 and extends inward and interacts with VP6, which

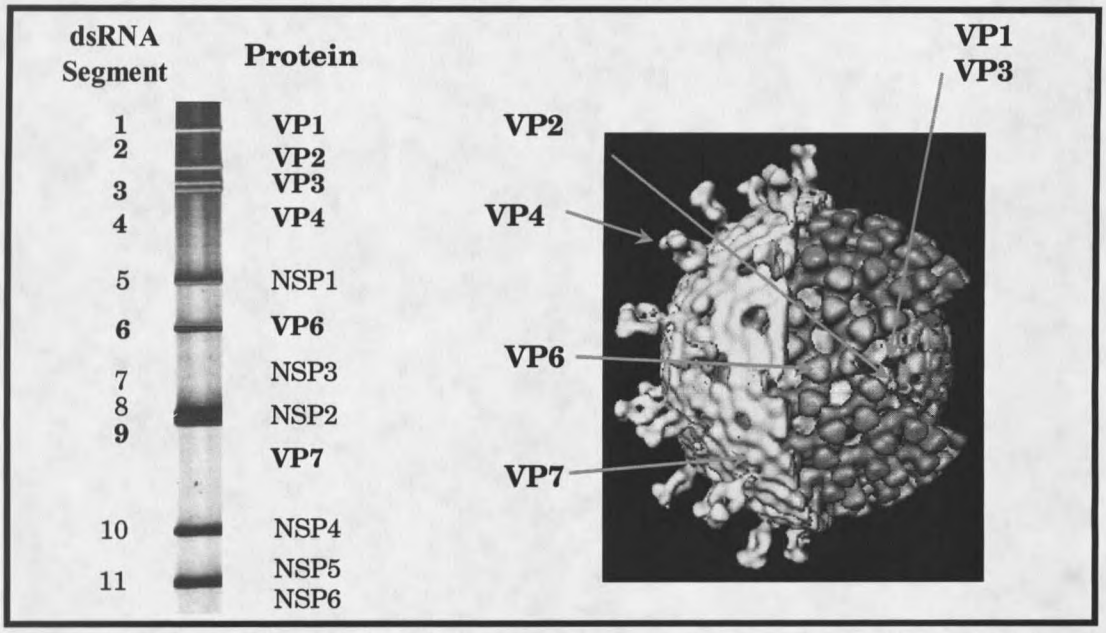

Figure 1.1: Structure of the rotavirus particle and viral proteins. On the left is a polyacrylamide gel showing the eleven segments of dsRNA and the proteins encoded by each gene. A three-dimensional computer reconstruction of the complete virus particle is on the right. Part of the outer and middle protein layers have been removed in order to see the middle and inner layers. Revised from Estes [106].

makes up the inner capsid [105, 107, 108]. VP6 surrounds the core and imparts long-term stability to the particle [109]. The core layer is formed by VP1, VP2, and VP3 and encapsidates the viral genome [110-112]. The rotavirus genome consists of 11 segments of double stranded (ds) RNA [113]. Most segments are monocistronic with the exception of segment 11 [114, 115]. These 11 segments code for 12 proteins, six structural and six nonstructural [106, 115]. Structural proteins, denoted as VP, are present in the mature virion, while nonstructural proteins, denoted NSP, are needed for genome replication in infected cells but are not present in mature virion.

The sizes of the RNA genome segments vary from 3302 nucleotides of segment 1 to 663 nucleotides of segment 11 [116, 117]. There are several common features among the rotavirus RNA segments. Transcribed viral mRNAs contain a 5' cap structure (m^7GpppG^m), but no polyadenylated tail, and at the extreme 3' and 5' termini there are consensus sequences among the genome segments [118]. The 5' terminal consensus sequence is 5'(GGC(A/U)$_7$) and the 3' consensus sequence is 5'((A/U)U(U/G)(U/G)GACC)3' [118, 119]. In the consensus sequence, more heterogeneity exists between different segments of a single strain than between homologous segments of different strains [120]. The 5' and 3' untranslated regions (UTR) are short, but vary in length and sequence between the different segments (Table 1.1) [25]. All 5' UTR are less than 50 bases long, and the 3' UTR can be between 17 and 182 bases with the exception of the second open reading frame of gene segment 11, which contains 5' and 3' UTRs of 79 and 291 bases, respectively [121] .

Table 1.1: UTR lengths for the gene segments [106, 117]

Gene Segment	Protein	5' UTR (length)	3'UTR (length)
1	VP1	18	17
2	VP2	16	28
3	VP3	49	34
4	VP4	9	22
5	NSP1	32	73
6	VP6	23	139
7	NSP3	46	59
8	NSP2	25	131
9	VP7	48	33
10	NSP4	41	182
11	NSP5	21	49
	NSP6	79	291

The entire 3' and 5' UTR are conserved between homologous segments of different strains [120]. Finally, all rotavirus mRNA must have a common *cis*-acting signal for the polymerase to bind because they all are replicated by the same polymerase [25, 122]. However, the mRNA segments also must contain a unique and likely separate signal for packaging because the mature virion contains only one copy of each dsRNA segment [25].

Rotavirus Replication Cycle

Binding and Entry. Rotavirus infects the apical cells of the villi of the small intestine and replication takes place within the cytoplasm of these cells [6]. A schematic of the replication cycle is shown in Figure 1.2. To enhance infectivity, VP4 is cleaved by trypsin into two subunits, VP5* and VP8*, which remain associated with the virion [123-125]. The initial trypsin cleavage event may be critical for conferring proper structural conformation to VP4 for subsequent proteolysis. The initial cleavage of VP4 by trypsin allows for recognition of other cleavage sites on VP5*, resulting in further cleavage of VP5*. This additional cleavage of VP5* increases the infectivity of the virus [126].

The virion attaches to the cell by VP4 or its cleavage products [127], and so VP4 is considered the major cell attachment protein [128]. VP8* mediates viral attachment to the cell via sialic acid [129], while VP5* initiates viral attachment in a sialic

independent manner by binding to other receptors such as the integrin α2β1 [128]. Most rotavirus strains initiate infection of cultured cells by sialic-acid independent mechanisms [130].

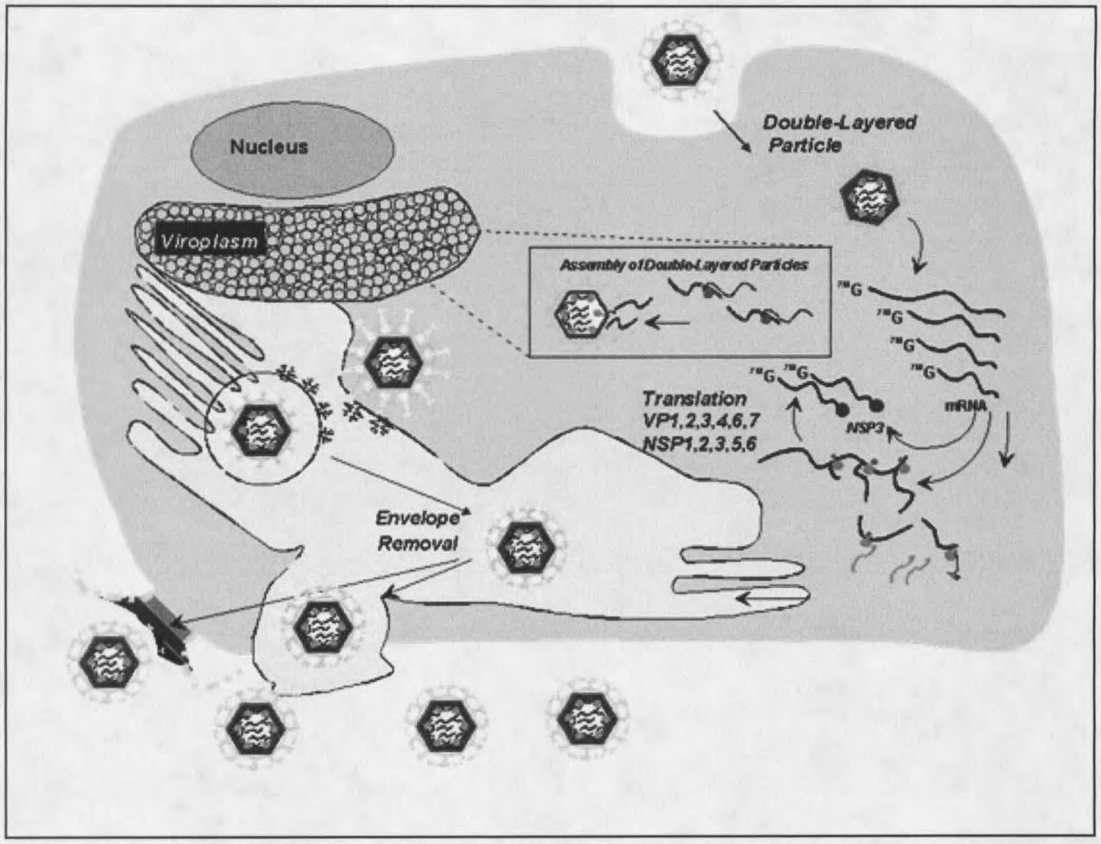

Figure 1.2: Major features of the rotavirus replication cycle. These features include: i) adsorption and penetration of the virus particle into the cell, ii) messenger RNA production in the cytoplasm from the double layered particles, iii) translation of the mRNA into proteins iv) rReplication and packaging in the viroplasm, v) maturation of the virus particle in the ER, and vi) release of the mature virion via cell lysis. Modified from Estes [106].

Besides functioning in cell attachment, VP5* also permeabilizes the cell membrane, allowing for rapid virus entry into the host cell [131, 132]. The mechanisms for permeabilization are unknown, but VP5* contains a hydrophobic fusion domain

which has sequence similarity with fusion domains of proteins from other viruses, such as the E1 protein of the Sindbis and Semliki forest viruses [131]. One possibility is that VP5*-cellular membrane interactions form transient pores that allow for size-selected permeabilization. Once the virus is attached, it is internalized in 60-90 minutes [133]. The internalization of the virus requires active cellular processes because the virus will attach but not penetrate the cell at 4°C [133, 134]. The virus enters the cell by penetrating the cellular membrane [135, 136]. During the penetration, the outer capsid consisting of the VP4 and VP7 is disrupted, leaving the transcriptionally active double-layered particle in the cytoplasm of the cell [137].

Transcription and Translation. Since no cellular proteins are capable of transcribing or replicating a dsRNA template, rotavirus encodes a RNA-dependent-RNA polymerase (VP1) and a guanyltransferase (VP3) [122, 138-140]. VP1 and VP3 interact with VP2 at the five-fold axis of the core [112, 141]. Transcription occurs near the five-fold axes of the core and each genome segment is thought to be transcribed by a specific polymerase complex. To exit the double-layered particle, the growing transcripts are translocated across the intact capsids through channels adjacent to the site of synthesis [105, 141]. This suggests that multiple RNA segments can be simultaneously transcribed, and therefore, viral transcripts can be released concurrently from an actively transcribing particle [142]. Efficient mRNA production occurs only within the context of a fully intact double-layered particle [137, 143]. All transcripts

are full-length positive strands made from the dsRNA negative strand. The mRNA is then either translated into viral proteins or transported to the viroplasm for packaging and replication.

Replication and Maturation. Viroplasms are the site for replication and assembly of double-layered particles [6, 144]. In the viroplasm, the 11 viral mRNA associate with the core replication intermediate, consisting of VP1, VP2, VP3, NSP2, NSP5 [145]. Synthesis of dsRNA occurs simultaneously with the packaging of mRNA templates into the core replication intermediate [146]. The decrease in particle size during replication suggests that the positive strand RNA template passes from the exterior to the interior of the replication intermediate [147]. As this occurs, the mRNAs act as templates for synthesis of the negative strand RNA, leading to the formation of dsRNA [147-149]. The dsRNA is resistant to digestion with dsRNA-specific Rnases, suggesting that the production of dsRNA occurs within the core. Furthermore, dsRNA is not found unprotected in the cytoplasm [150]. As previously mentioned, VP1 is the viral RNA-dependent RNA polymerase, but VP1 only exhibits replicase activity when interacting with VP2. NSP2 contains a helix destabilizing property that unwinds the mRNA prior to replication and packaging [151]. NSP5 crosslinks to the VP1/ VP2 complex and to NSP2. This suggests that NSP5 acts as bridge between NSP2 and the replication complex (VP1, VP2, and VP3) [152, 153]. The fact that NSP5 dislodges VP6 from purified double-layered virus-like particles consisting of VP2 and VP6, suggests that NSP5 may block or delay the assembly of the outer capsid, allowing the core replication intermediate to maintain the replicase activity [152]. Before the

replication intermediate leaves the viroplasm VP6 binds to the core replication intermediate, giving rise to the double-layered particle [134, 146, 154].

The 3'-consensus sequence is essential for minus-strand synthesis because viral transcripts lacking the 3' consensus sequence no longer serve as templates for the synthesis of dsRNA in vitro [155]. Furthermore, when the 3' consensus sequence is placed on foreign RNA, the foreign RNA can serve as a template for the synthesis of dsRNA in vitro [155, 156].

After replication and packaging, one dsRNA for each 11 gene segment is found within the double-layered particle. The mechanism of selective packaging for each segment is unknown, and none of the rotavirus RNA-binding proteins is known to have the ability to distinguish between the different viral RNAs. This suggests that selective packaging is not mediated by viral proteins alone, and therefore, other mechanisms have been proposed [157]. One mechanism suggests selective packaging could be mediated by an RNA-RNA interaction occurring in *trans* between viral mRNA templates. These RNA-RNA interactions are then stabilized by the rotavirus RNA-binding proteins. The RNA-binding proteins could also alter the structure of the mRNA template to make the RNA packaging sites sterically accessible [157].

Once in the cytoplasm, the double-layered particle would either synthesize more mRNA or would undergo maturation through the endoplasmic reticulum (ER). The double-layered particle buds through the ER and becomes transiently enveloped [25]. As the particles continue to move through the ER, the envelope is lost and the two outer

capsid proteins (VP4 and VP7) condense around the particle to form the mature virion [25]. The triple-layered particle is then released through cell lysis [154].

Regulation of Eukaryotic Gene Expression

Eukaryotic Translation

Protein synthesis is a fundamental step in gene expression and a crucial control point for regulation. Regulating translation allows a cell to rapidly respond to environmental stimuli without the need for transcription, processing, or transport of new mRNA [158]. Translation is a multistep process that includes initiation, elongation, and termination phases.

Translation initiation is divided into three steps (Figure 1.3). First, is formation of the 43S preinitiation complex. The initiator tRNA (Met-$tRNA_i^{met}$), a specific tRNA derivative used to bind to the start codon and initiate protein synthesis, is selected by eukaryotic initiation factor (eIF) 2. EIF2 is bound to GTP and when it binds to the initiator tRNA it forms a stable ternary complex (eIF2-GTP-met-$tRNA_i^{met}$) [159]. The ternary complex binds to the 40S ribosomal subunit to form the 43S preinitiation complex. This reaction is stimulated by eIF3 and eIF1A which are associated with the 40S subunit [160]. These two initiation factors are thought to help promote dissociation of the 80S ribosome into the 40S and 60S subunits [160].

Binding of the 43S preinitiation complex to the mRNA is promoted by eIF4B, eIF3, and eIF4F, a heterotrimeric complex consisting of eIF4E, eIF4G, and eIF4A [158, 160]. EIF4E, also known as the cap-binding protein, is responsible for binding to the 5'