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
Montana State University


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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A thesis submitted in partial fulfillment
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APPROVAL

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Dana Nicole Mitzel

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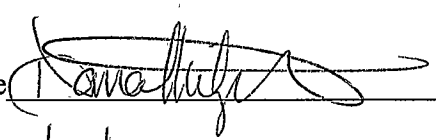
Signature 

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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

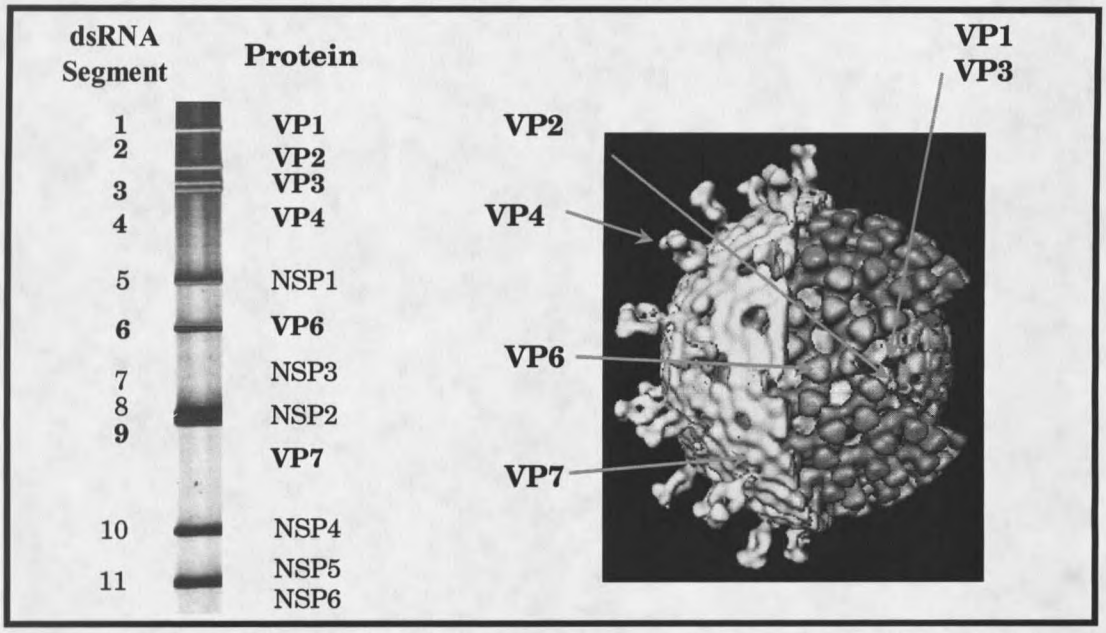



Figure 1.1: Structure of the rotavirus particle and viral proteins. On the left is a polyacrylamide gel showing the eleven segments of dsRNA and the proteins encoded by each gene. A three-dimensional computer reconstruction of the complete virus particle is on the right. Part of the outer and middle 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].

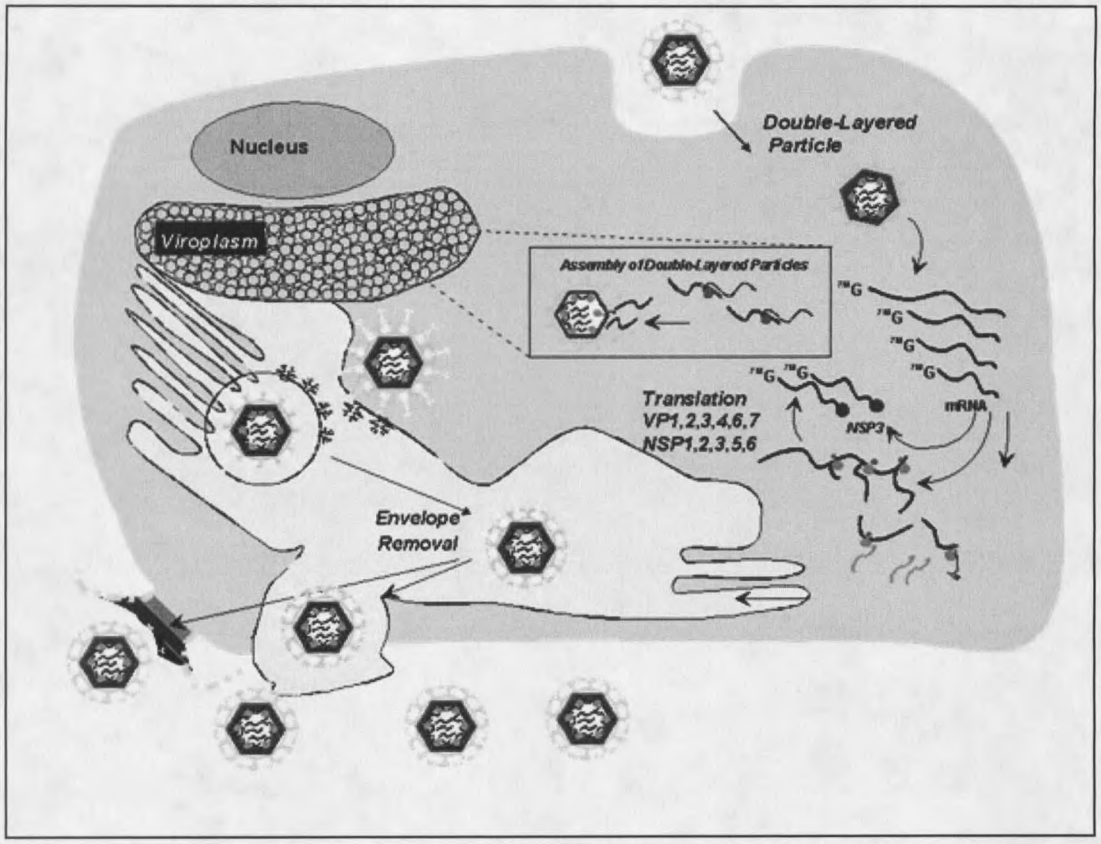



Figure 1.2: Major features of the rotavirus replication cycle. These features include: i) adsorption and penetration of the virus particle into the cell, ii) messenger RNA production in the cytoplasm from the double layered particles, iii) translation of the mRNA into proteins iv) rReplication and packaging 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'

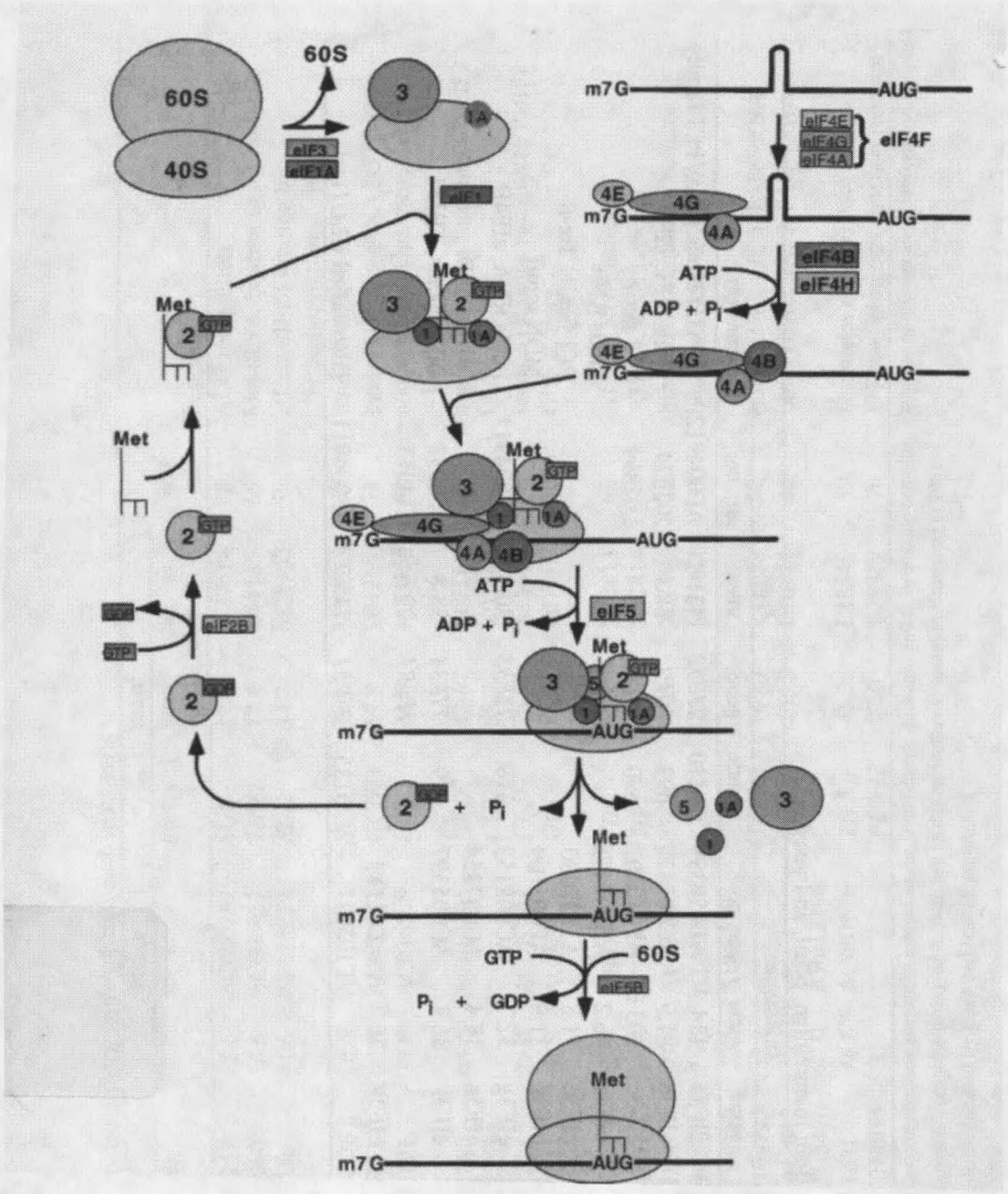



Figure 1.3: Eukaryotic Translation Initiation. Initiation factors are shown as labeled circles and appear as they are first implicated in the pathway. The pathway shows formation of the ternary complex, 43S preinitiation complex, translocation of the ribosome to the start codon, and binding of the 60S subunit [164].

terminal $m^7GTP$ cap found in most eukaryotic mRNA [161]. EIF4E is the limiting initiation factor in cells, and therefore plays a role in regulating translation by discriminating between mRNAs [162]. The eIF4A subunit present in eIF4F contains RNA helicase activity and can unwind RNA in the absence of eIF4E or eIF4G [163]. However, the helicase activity is stronger in the eIF4F complex and is further enhanced by eIF4B [164]. Once the secondary structures in the mRNA are removed, the 40S subunit binds to the mRNA [160]. The binding of the 40S subunit to the mRNA occurs through an interaction between eIF4G and eIF3 [165]. The 43S initiation complex translocates the 5' untranslated region (UTR) in a 5' to 3' direction searching for the start codon (AUG) in the correct context [166, 167]. The ribosome stops when it reaches the initiation codon, primarily through the RNA-RNA interaction of the AUG codon in the mRNA and the CAU anticodon of the bound met-$tRNA_i^{met}$ [168]. Finally, upon the AUG codon recognition, the initiation factors dissociate from the 40S subunit and allow joining of the 60S subunit to form the actively translating 80S ribosome [169]. Now that the active ribosome is associated with the start codon, elongation ensues.

Elongation is the phase of translation in which the polypeptide is assembled (Figure 1.4). This process is assisted by several elongation factors (eEF). EEF1A and eEF1B are used in aa-tRNA recruitment and eEF2 is used in the translocation of the ribosome down the mRNA [170, 171]. At the end of initiation, the initiator tRNA is in the P site, leaving the aa-tRNA binding site (A site) available for an incoming aa-tRNA [158]. This serves as the start of elongation. During the elongation phase of protein

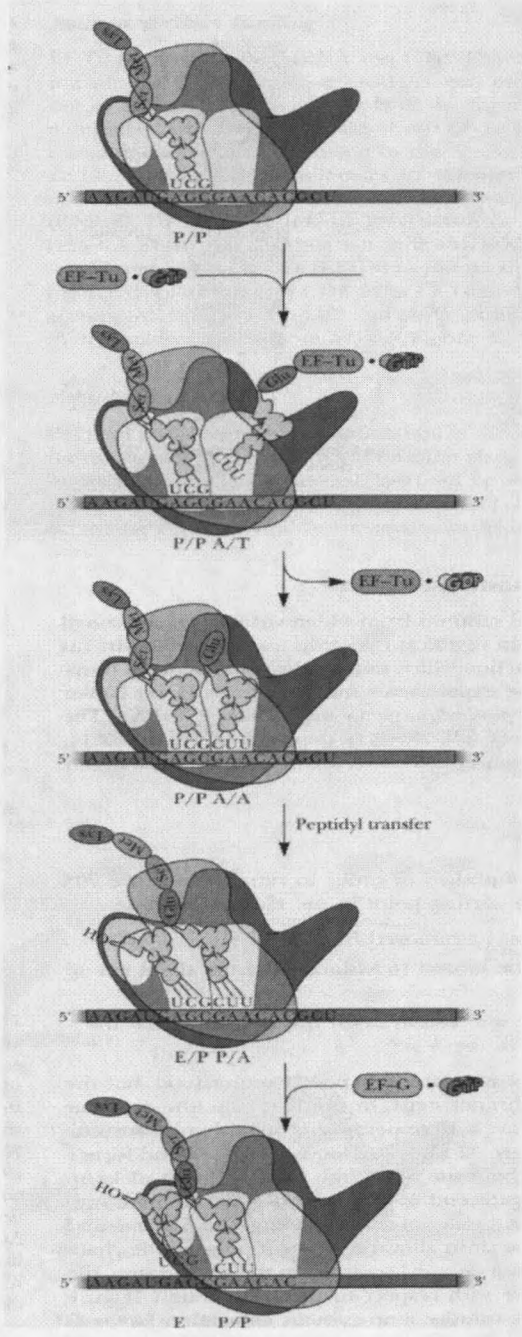



Figure 1.4: Schematic for the movement of tRNAs during translation elongation. EF-Tu represents eEF1A and EF-G represents eEF2. The A, P, E, sites are represented by the light blocks going from right to left respectively [171].

synthesis, eEF1A binds to GTP. When eEF1A is bound to GTP it is then able to bind aa-tRNA to form a ternary complex (eEF1a-GTP-aa-tRNA) [170]. The aa-tRNA is brought to the A site of the ribosome when bound in this ternary complex [170]. The correct codon/anticodon interaction results in a conformational change in the ribosome, which stabilizes tRNA binding and triggers GTP hydrolysis by eEF1A [172, 173]. The hydrolysis of GTP leads to the release of eEF1A-GDP from the ribosome [170]. EEF1A-GDP is recycled to the active eEF1A-GTP form by eEF1B, which acts as a guanine nucleotide exchange factor [174]. The tRNA then enters the peptidyl transferase site and a peptide bond is formed between the incoming amino acid and the peptide found in the P-site [172]. Peptide bond formation involves the deacylation of the P-site tRNA and the transfer of the peptide chain to the A-site tRNA. Following peptidyl transfer, the ribosome contains a deacylated tRNA in the P-site and the peptidyl tRNA in the A-site [173, 175]. Translocation of the tRNA and the ribosome is facilitated by eEF2. EF2 enters the A-site, forcing the peptidyl tRNA out of the A-site and into the P-site, while at the same time the deacylated-tRNA moves into the exit (E) site [176]. By translocating the peptidyl-tRNA into the P-site and by preserving the tRNA/mRNA contacts, the mRNA moves three nucleotides downstream, leaving the A-site empty and ready to receive a new ternary complex [173].

The presence of a termination codon (UAA, UAG, UGA) in the A site of the ribosome signals polypeptide chain release (Figure 1.5) [173, 177]. The release of the polypeptide does not depend on a tRNA molecule, but does require two classes of release factors (eRF). The first class of release factors contains eRF1, which recognizes

the stop codon, possibly through tRNA mimicry [178]. The second class of release factors (eRF3) recycles eRF1 in a GTP-dependent manner [179]. The three termination codons are decoded by eRF1 [180]. ERF1 promotes ribosome-catalyzed peptidyl-tRNA hydrolysis of the ester bond of the peptidyl-tRNA molecule located in the P site, thereby releasing the nascent peptide chain [178]. After peptide release, eRF3 catalyzes the dissociation of eRF1 from the A-site [181]. To remove eRF1 from the ribosome, eRF3-GTP complex enters the ribosome and interacts with both eRF1 and the ribosome to induce GTPase activity. Upon GTP hydrolysis, eRF1 is released from the ribosome and ribosomal subunits are dissociated [182].

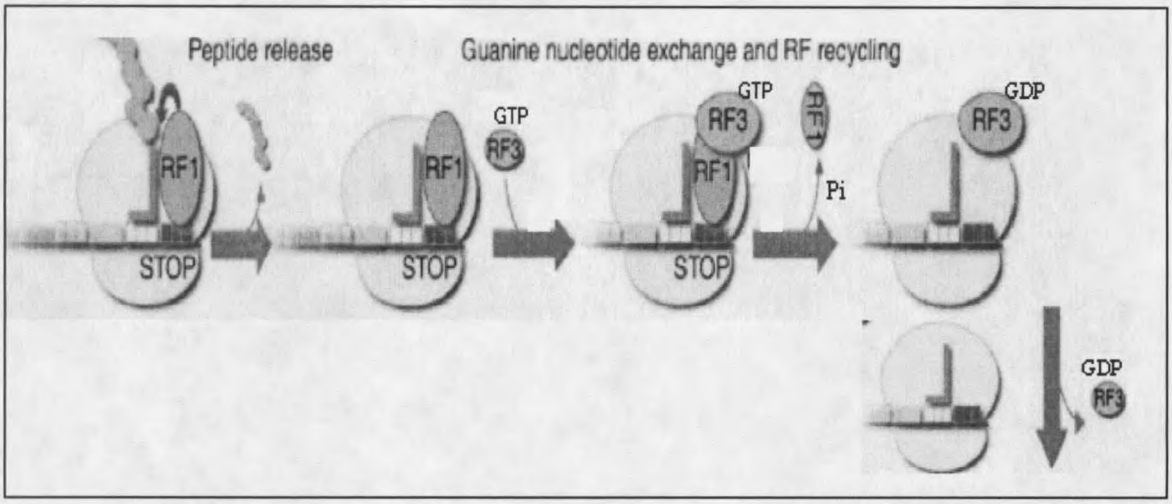



Figure 1.5: Eukaryotic translation termination. Release factors are shown in labeled circles. Release factor eRF1 promotes peptide-chain cleavage, thereby releasing the peptide. eRF3 catalyzes the dissociation of RF1 from the A-site. Modified from Nakamura [177].

Translational Control in Eukaryotes

Translational control is the idea that synthesis of a protein may be regulated within a cell and the relative rates of protein synthesis may be altered [183]. Regulation can be found at the different levels of protein synthesis (initiation, elongation, termination), however, in the majority of the cases, translational control occurs at the level of initiation [184]. Eukaryotic mRNAs have several features that play important roles in their translational efficiencies, such as length and secondary structures in the UTRs, polyadenylated (poly(A)) tails and cap structures. The length and secondary structures of the 5' UTR regulate translation in different ways. Long 5' UTRs impede initiation by influencing scanning of the translation machinery due to the increased likelihood of secondary structures and upstream open reading frames [185, 186]. However, while long, unstructured 5' UTR enhances translation [187], short 5'UTRs (less than ten nucleotides) decrease the efficiency of translation. This is likely due to the ribosome not uniquely recognizing the 5' proximal start codon, which leads to the initiation at a downstream initiation codon [187, 188].

Secondary structures within the 5' UTR could either impede or enhance initiation. If the 5' UTR contains strong secondary structures, initiation is impeded by blocking the translocation of the ribosome [166, 189]. Secondary structures near the cap also inhibit initiation by preventing the 40S ribosomal subunit from binding to the cap structure on the mRNA [166]. However, having a stable hairpin structure twelve nucleotides downstream of the initiation codon enhances initiation. This structure

instigates a pause in the scanning of the ribosome at the start codon allowing for efficient interaction between the codon and the anticodon [190].

Both the 5' UTR and the 3' UTR contain *cis*-acting sequences and structures that interact with *trans*-acting factors that regulate translation [191-196]. *Cis*-acting elements are usually sequences and secondary structures in the RNA. *Trans*-acting elements are usually proteins and include the general translational machinery and regulatory proteins that interact with *cis*-acting sequences of the mRNA.

The placement of the start codon also affects the efficiency of translation. According to Kozak, a strong initiation sequence context surrounding the AUG contains a purine three nucleotides upstream of the A in the start codon and a G three nucleotides downstream of the A in the start codon [167, 197]. If the start codon deviates from this context, it can be passed over during translocation of the ribosome, and initiation would start at a downstream AUG in a more favorable context. This is a process called leaky scanning and can lead to the production of two proteins from one mRNA [166]. Typically in eukaryotic cells, the 5' proximal AUG serves as the initiation codon in 90% of the mRNA, even if the start codon is in a weaker context than a downstream AUG [167, 187]. The presence of an upstream AUG from the major initiation codon can reduce the translation efficiency from the major start codon [166, 198]. Some of the ribosomes initiate translation from the upstream start codon instead of the major start codon, decreasing the amount of protein synthesized from the major start codon [166].

Most eukaryotic mRNAs contain a 5' 7-methyl-guanosine cap structure ($m^7$GpppX) and a 3' poly(A) tail [199, 200]. Both the cap and the poly(A) tail confer RNA stability and protect the mRNA from degradation [199, 201, 202]. These two features of mRNA also have a synergistic effect on translation and therefore play an important role in regulating gene expression [203-209]. The cap structure binds to the translation initiation factor eIF4F and the poly(A) tail interacts with the poly(A) bindig protein (PABP) [158, 160, 203]. However, the PABP also interacts with the N-terminus of eIF4G [210, 211]. By binding simultaneously to the poly(A) tail and to eIF4G, the 5' and 3' ends are brought together, causing the mRNA to become circularized and enhancing translation [212]. Circularization likely enhances mRNA stability by maintaining the poly(A) tail and cap structure, and enhances translation by stimulating reinitiation by increasing the local concentration of ribosomes [201, 213, 214].

Regulation of Host Cell Translational Apparatus by Viruses

Viruses do not encode or carry the machinery for protein synthesis and are therefore dependent on the host cell translation machinery [215]. To reduce the impact of this dependency, viruses have developed ways to facilitate the translation of viral mRNAs over host cell transcripts. Although some of these alternative mechanisms of protein synthesis are now identified for host cell mRNAs, viruses appear to have a greater range and diversity of tactics in translating viral mRNA [215]. These mechanisms supercede cellular mRNA translation through the recruitment and or modification of translation factor function, unconventional translational mechanisms, and host shutoff.

Host shutoff is the interference with the production, maturation or stability of cellular DNA, RNA or proteins. Shutoff of host protein synthesis requires the virus to compromise some aspect of the translation system while at the same time engineering mechanisms to bypass these compromised events. Shutoff allows the virus to seize the cellular machinery by alleviating competition between viral and cellular mRNA. Selective translation of viral mRNA over endogenous host transcripts can be attributed to several factors. These factors include viral perturbation of intracellular ion concentrations, nucleotide metabolism, alteration in RNA stability, processing, or export, and the recruitment of host factors [216-219]. Selective translation during host shutoff could also be due to viral mRNA outcompeting host mRNA for the translational machinery [215]. When competing for translational machinery, the preferential synthesis of viral proteins hinges on the differences between viral and host cell mRNAs [215]. These differences can be enhanced by virus encoded proteins or by alterations in the translational machinery that put translation of viral mRNAs at an advantage over cellular mRNAs.

Altering the cellular translational components allow the viral mRNA to be preferentially translated. Usually these modifications include cleavage or covalent modification of the translation factors. One such mechanism blocks the phosphorylation of eIF2α, a subunit of eIF2 [220]. Phosphorylation of eIF2α blocks the recycling of GDP for GTP and prevents formation of the ternary complex, thereby, blocking initiation [215, 221]. In a virus-infected cell, eIF2α is phosphorylated by the double-stranded RNA dependent protein kinase R (PKR) [222]. PKR is

transcriptionally induced by interferons in virus infected cells and becomes activated by binding to dsRNA or ssRNA with extensive secondary structures [222, 223]. Activated PKR phosphorylates eIF2α and inhibits mRNA translation [222], and so to avoid the deleterious effect this would have on viral mRNA translation, viruses have devised strategies to block the different parts of the PKR pathway. Some of these mechanisms include blocking the dsRNA binding domain or blocking the interaction between PKR and eIF2 [223-227].

Another way to affect initiation of cellular mRNA is by proteolytic cleavage of eIF4G. eIF4G is an important translation initiation factor in cap-dependent mRNA translation because it acts as a molecular bridge for the assembly of the cap-binding complex upon the 5' end of the mRNA and facilitates the interaction of this complex with the 43S preinitiation complex [158]. Cleavage of eIF4G by picornavirus proteases separates it into two terminal cleavage products, the amino terminus, which interactes with eIF4E and PABP, and the carboxyl domain, which interactes with eIF4A and eIF3. Under these conditions, a functional eIF4F complex is unable to form and cap-dependent initiation is inhibited [228, 229]. This mechanism gives viruses that initiate translation through an internal ribosome entry site, such as picornaviruses, an advantage over cellular mRNA in competing for the cellular translational apparatus. By altering these initiation factors, viruses have devised mechanisms to surpass these restrictions, thereby placing the viral mRNA at an advantage for translation.

Virus-Encoded Mechanisms of Translational Control/Initiation

Viruses usurp the translation machinery of the host cell by unique ways of translating the viral mRNA. Although some mechanisms (e.g. IRES) are well described, most remain incompletely characterized. Such mechanisms include internal ribosome entry sites, shunting, frameshifting, and leaky scanning. Some examples of unique mechanisms are outlined below.

Certain viruses, such as picornaviruses, pestiviruses, and hepaciviruses, initiate translation through a cap-independent mechanism via an internal ribosome entry site (IRES) [230-232]. These viruses contain a long 5'UTR with extensive secondary structures making the UTR incompatible for the ribosome to translocate the UTR in search of the start codon [187]. However, these secondary structures allow for the assembly of the translational machinery on or near the initiation codon by direct ribosome recruitment through protein:RNA interactions [232]. Other viruses such as cauliflower mosaic virus and adenovirus have evolved mechanisms such as ribosome shunting to enhance cap-dependent translation of the viral mRNAs over the cellular mRNAs. Ribosome shunting is a mechanism that requires cap-dependent initiation, translocation of the ribosome along the 5' UTR, followed by an internal initiation [233]. The ribosome translocates the 5' UTR until a *cis*-acting shunting sequence is recognized. This shunting element promotes translocation of the ribosome to a downstream receiving element, allowing the ribosome to bypass a large portion of the 5' UTR and to initiate translation at a downstream start codon [233]. Similar to IRES mediated translation, ribosome shunting avoids problems with scanning of a highly

structured 5' UTR. Shunting also reduces the need for the host eIF4F helicase activity to melt secondary structures within the mRNA that might impede the normal translocation [215]. This facilitates the selective translation of viral mRNA under the competitive conditions when eIF4F is limiting.

In eukaryotic cells, the majority of mRNA are translated from the 5' proximal AUG. However, in rare cases, the mRNA contains a short upstream open reading frame [234]. Many viruses, including human immunodeficiency virus and mouse mammary tumor virus, take advantage of this by synthesizing a bicistronic mRNA that encodes two different proteins. Frame-shifting and leaky scanning are widely used by viruses and allow for the translation of multiple open reading frames from a common mRNA substrate [215]. Since each open reading frame does not need to be in the same reading frame, the usefulness of these mechanisms is apparent. Leaky scanning of the bicistronic mRNA allows for coordinate expression of the viral proteins [235]. Usually the first AUG is in a suboptimal context, and therefore is a weak initiator. This allows for the slow accumulation of the protein encoded by the first open reading frame to levels needed for its function during the late stage of infection. The first open reading frame can regulate the synthesis of the second protein by impeding ribosome scanning to the downstream open reading frame. This allows for expression of the second protein to coincide with the protein's function in late-stage replication [215, 235]. Likewise, ribosomal frameshifting allows the translating ribosome to shift position upstream or downstream by one nucleotide, resulting in a change in the reading frame [236]. The frameshift sites within the viral mRNA correspond to a heptanucleotide

sequence in which the mRNA slips one base with respect to the tRNA in the A-site and P-site of the translating ribosome [236, 237]. A psuedoknot six nucleotides downstream stimulates frameshifting by pausing the ribosome with the heptanucleotide sequence in the decoding site. The psuedoknot plays an important role in stimulating a frameshift event, possibly by interacting with *trans*-acting factors [237]. These methods allow for an increased coding capacity of the viral genome and allow for the synthesis of two or more viral proteins when the translation machinery is in limited amounts [215].

## Regulation of Rotavirus Gene Expression

### Transcriptional and Translational Control

Johnson and McCrae showed that there is regulation of rotavirus gene expression at the level of transcription and translation [29]. These data show that even though there are equimolar concentrations of dsRNA present in the double-layered particle, the viral mRNAs are produced in non-equimolar concentrations. Furthermore, the level of the mRNA for a gene does not reflect the amount of corresponding proteins produced, as shown in Table 1.2 [29]. These data suggest that the viral mRNAs contain signals that regulate levels of protein synthesis. The primary sequence and the secondary structures of the UTRs are predicted to bind viral proteins, cellular proteins or both. This suggests that the UTRs may play several roles in virus replication, including the regulation of protein synthesis, mRNA transport, and packaging [238].

Table 1.2: Analysis of levels of transcription and translation for the rotavirus genes at 6.5 post infection. [a]relative to NSP5 [29].

| Protein/gene | Production of proteins[a] | Accumulation of mRNA[a] |
|---|---|---|
| VP1/ gene segment 1 | 0.15 | 1.3 |
| VP2/ gene segment 2 | 1.32 | 2.3 |
| VP3/ gene segment 3 | 1.27 | 1.3 |
| VP4/ gene segment 4 | 1.27 | 0.6 |
| NSP1/ gene segment 5 | 1.57 | 0.9 |
| VP6/ gene segment 6 | 25.02 | 0.7 |
| NSP3/ gene segment 7 | 10.68 | 2.8 |
| NSP2/ gene segment 8 | 3.48 | 0.9 |
| VP7/ gene segment 9 | 4.43 | 0.9 |
| NSP4/ gene segment 10 | 38.65 | 0.7 |
| NSP5/ gene segment 11 | 1 | 1 |

There are three patterns of temporal control of synthesis of rotavirus mRNA [29]. Messenger RNA synthesis first proceeds at a linear rate throughout the infection cycle. This pattern is observed for genes 1, 2, 6, 8, and 11. In contrast, the mRNA for gene 7 accumulates rapidly during the early part of the infection, then reaches a plateau, where it remains throughout the growth cycle. In the third pattern, the mRNAs for genes 3, 4, 5, 9, and 10 accumulate slowly during the early phase of infection and the rate increases in the latter part of the infection [29]. Early patterns of transcription can be preserved until late in infection in the presence of a protein synthesis inhibitor, suggesting that some of the early gene products may play a regulatory role in replication [29].

Even though there is no distinct early to late switch in viral protein synthesis, quantitative variation in the kinetics of synthesis of the different viral proteins does

occur during the infection. Once again, three patterns are apparent. These patterns consist of proteins that reach maximum rates of synthesis early in the infection (e.g. VP7), at approximately four hours post infection (e.g. VP1), or the levels of protein synthesis continue to increase throughout the infection (e.g. VP6) [239].

Although the mechanisms for the regulation of rotavirus gene expression are not well understood, several factors such as competition, *cis*-acting sequences, and the UTRs could function in the translational control of the viral genes. *Cis*-acting signals present in the 5' and 3' UTR can contribute to the regulation of gene expression. One such sequence is the conserved four nucleotide sequence (GACC) present at the 3' end of the rotavirus mRNA [240]. This sequence binds to NSP3 and enhances expression of a luciferase reporter gene in vitro [240, 241]. The length of the 3' UTR also could be responsible for the enhanced translation of some genes over others. The length of the 3' UTR is shown to correlate with the translational efficiency of reporter genes lacking a poly(A) tail. The longer the 3'UTR, the more efficiently the gene is translated [242].

Competition between the mRNAs for the translational machinery is known to function in the translation efficiency of the gene [215]. Therefore, the rotavirus mRNAs can compete with each other for the translational machinery. The ability of the mRNA to competitively interact with the translational machinery can be due to the *cis*-acting sequences present in the mRNA and the *trans*-acting proteins, which interact with these sequences. These *trans*-acting proteins can inhibit or enhance the recruitment of the ribosome to the mRNA and thereby increasing or decreasing

rotavirus gene expression [243]. Limited evidence exists for the participation of *cis*-acting sequence and *trans*-acting factors in regulating expression of rotavirus genes.

Viral Proteins Involved in Rotavirus Translational Control

NSP3 is a 36 kDa protein encoded by gene segment 7 and is expressed at moderate levels in infected cells [29]. Unlike the majority of rotavirus proteins which are found in the viroplasm, NSP3 is found diffuse in the cytoplasm and is associated with the cytoskeleton [244, 245]. Sequence analysis suggests two major functional domains present in NSP3. The first domain is a highly conserved basic region (aa 83-150) that is predicted to have an alpha-helical structure. This domain interacts with the group-specific 3' end consensus sequence of rotavirus mRNA in vitro and in vivo [241, 246, 247]. The second domain is found in the carboxyl-half of the protein and contains heptapeptide repeats of hydrophobic residues. These heptad repeats are shown to be responsible for the dimerization of NSP3 [244].

Recent studies showed that NSP3 enhances translation of viral genes by simultaneously binding to both the 3'end of viral RNA and to eIF4G [248]. The C-terminal domain of NSP3 outcompetes PABP for the same binding domain on eIF4G [210, 249]. These data suggested that NSP3 enhances translation of viral mRNAs by acting as a PABP analogue that promotes circularization of the viral RNA and by inducing host cell shutoff [248-251].

Gene segment five encodes NSP1, a 53 kDa protein expressed early in infection and at low levels [29, 252]. Similar to NSP3, NSP1 is located in a punctate-like manner throughout the cytoplasm, and is associated with the cytoskeleton [245, 253]. Using the

two-hybrid system, an interaction between NSP1 and NSP3 is detected and recorded as the strongest interaction among the nonstructural proteins [254]. NSP1 is the least conserved rotavirus protein, but does contain a highly conserved cysteine-rich region in it's amino-terminal half [255, 256]. Two cysteine-rich regions have characteristics of zinc finger motifs. The first motif is present in the N-terminus and contains the following sequence: Cys-$X_2$-$Cys_2$-$X_8$-Cys-$X_2$-Cys-$X_3$-H-X-C-$X_2$-C-$X_5$-C where X represents any amino acid other than cysteine [238]. This first zinc finger is highly conserved throughout all group A rotavirus strains. The second zinc finger (HIS- $X_2$-Cys- $X_6$-Cys-$X_2$-Cys) is located at residues 315-328 and is only found in a few strains [257]. Zinc finger domains are common features found in many regulatory and nucleic acid binding proteins, such as the transcription factor TFIIIA [258, 259]. NSP1 has specific affinity for all 11 rotavirus mRNAs [245]. The RNA binding domain of NSP1 is mapped to the cysteine rich region and the zinc finger domains are essential for the binding activity [245]. Binding of NSP1 to viral mRNA occurs within the first 278 nucleotides of the 5' end [145, 245].

Gene segment five rearrangements have been found in several strains of rotavirus. These strains are unable to produce full-length NSP1, but are capable of growing in cell culture. However, plaques formed by the mutant virus are significantly smaller than plaques produced by wildtype virus [260]. These observations suggest that even though NSP1 is not essential for replication, it may play a beneficial role in replication.

## Rationale and Aim of Research

The molecular mechanisms and the proteins that regulate transcription and translation of rotavirus genes are not well defined. It is known that there is transcriptional and translational control of rotavirus gene expression. Equimolar concentrations of dsRNA are present in the double-layered particle, but viral mRNAs are produced in nonequimolar concentrations. Furthermore, the amounts of specific rotavirus proteins do not correlate with the amount of the cognate mRNAs. Temporal and quantitative control of rotavirus gene expression has also been demonstrated. This suggests that the viral mRNAs contain signals that regulate the levels of protein synthesis. Studies have shown that *cis*-acting sequences within the 5'UTR of viral mRNAs play an important role in regulating translation by binding to *trans*-acting factors. Based on the studies outlined above, we hypothesize that NSP1 is involved in regulating rotavirus gene expression through interactions with viral mRNA.

In chapter three of the text, we investigated possible mechanisms of regulation of rotavirus gene expression. To carry out this analysis, we measured the accumulation of viral mRNA, the stability of viral proteins, and the translation efficiency of viral mRNA encoding different rotavirus genes. In chapter four, we examined the role of NSP1 in the regulation of translation of rotavirus mRNA by performing comparative polysome analyses between a mutant rotavirus strain that does not encode NSP1 and a wildtype strain. Together the results support the hypothesis that NSP1 may function in regulating rotavirus gene expression, perhaps at the level of protein synthesis.

# CHAPTER TWO

# MATERIALS AND METHODS

## Virus and Cells

Isolation and characterization of bovine rotavirus strains B641, NCDV, and A5-16 have been described [260, 261]. B641 was cultured in MA104 cells that were maintained in M199 medium (Irvine Scientific, Santa Ana, CA) in the presence of 5% fetal bovine serum (FBS, Atlanta Biologicals, Norcross, GA). Cells were infected at approximately 90% confluency at multiplicities of infection of 0.1 plaque forming units (pfu)/cell for stock virus preparation and 5-10 pfu/cell for RNA or protein analysis. The virus was activated by treatment with 5 μg/ml trypsin (>180 U/mg, Worthington Biochemicals, Freehold, NJ) for 30 minutes at 37°C. Activated virus was adsorbed to cell monolayers either at 4°C or 37°C depending on the experiment. Low temperature adsorption was performed to synchronize infections as much as possible by allowing binding of the virus to cells but preventing internalization [262]. Cultures were shifted to 37°C after a 1.5 hour adsorption, and harvested at indicated times under conditions specific to the experiment.

Cloning and Sequencing of B641 Gene 5

Gene 5 was cloned by RT-PCR. Transcriptionally active double-layered virus particles were purified by CsCl gradient centrifugation and viral mRNA was synthesized in vitro as previously described [263, 264]. Purified mRNA was reverse transcribed for three hours with avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) primed with the oligonucleotide 5'-cacggatccggtcacattttgcagggagtcttg-3' complementary to the 3' end of the gene 5 mRNA. Following reverse transcription, mRNA was degraded by alkaline hydrolysis, and cDNA was purified by ethanol precipitation in the presence of 0.3 M sodium acetate, pH 5.2. Gene 5 then was amplified with *Pfu* polymerase (Stratagene, La Jolla, CA) by 30 cycles of PCR with the above primer and a positive sense primer 5'-cacggatccggctttttttttatgaaaagtcttg-3'. Both oligonucleotides contained BamHI restriction sites for cloning (underlined) and were designed based on consensus sequence alignments of gene 5 sequences of other rotavirus strains. Cycling conditions were as follows: 94°C for 1 minute, 50°C for 45 seconds, and 72°C for 2 minutes. The resultant 1.5 kb PCR product was cloned into pBluescript KS+ (Stratagene, La Jolla, CA). The nucleotide sequence was determined on an ABI 310 Genetic Analyzer with BigDye Terminator™ chemistry. Comparison of the nucleotide sequence with those of other rotavirus strains indicated that gene 5 of B641 is 98% identical at the nucleotide level to gene 5 of bovine strain RF [265]. The nucleotide sequence has been deposited in GenBank and assigned accession number AF458087.

## Metabolic Labeling of Rotavirus Proteins

Viral proteins synthesized in infected cells were labeled as previously described with modifications [252]. MA104 cells were infected at a multiplicity of infection of 10 pfu/cell in medium lacking methionine and cysteine and containing 5% FBS. Fours hours post-infection, the medium was replaced with medium without FBS and containing 10 μg/ml actinomycin D and 40 μCi/ml TRANS$^{35}$S-label (ICN, Costa Mesa, CA). Cultures were harvested at indicated times by gentle rocking in RIPA buffer (150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 10 mM Tris-HCl pH 7.2, and 1% Trasylol). Labeled proteins were resolved by SDS-PAGE and visualized by autoradiography.

Pulse-chase labeling was performed as previously described [252] and as outlined above, except that following a 30 minute pulse with 40 μCi/ml TRANS$^{35}$S-label, cultures were chased with medium containing 400X unlabeled methionine and cysteine, and 100 μg/ml cycloheximide. Cultures were chased for indicated time points, harvested in RIPA buffer, and radiolabeled proteins were analyzed by SDS-PAGE. Radioactive protein bands were quantified on a BioRad Molecular Imager F-X with Quantity One software.

## RNA Extraction and Northern Hybridization

Total RNA was harvested from mock-infected or B641-infected cells by Trizol™ (Invitrogen-Life Technologies, Carlsbad, CA) extraction following the

procedures recommended by the manufacturer. RNA was electrophoresed in 1.2% agarose gels containing 6% formaldehyde and transferred to nylon membrane by capillary blotting. Radiolabeled probes were prepared by nick translation of specific gene fragments that were digested and purified from plasmid vectors. The plasmid containing SA11 gene 6 in the pSP65 vector (Promega, Madison, WI) was a gift from M.K. Estes, Baylor College of Medicine, Houston, TX [266]. The specific activity of the probes was determined by liquid scintillation counting, and care was taken to ensure the specific activities were comparable between probes for different genes and different experiments to allow quantitative interpretation of the data. Hybridizations were performed at 42°C in the presence of 50% formamide. Radioactive signals on the blots were quantified on a BioRad Molecular Imager F-X with Quantity One software.

## Polyribosome Analyses

All polyribosome analyses were performed several times and representative data are presented. Cytoplasmic extract preparation and polyribosome analyses were performed and interpreted as previously described [191, 267-270]. MA104 cells were mock infected or infected with the indicated rotavirus strain at a multiplicity of infection of 5-10 pfu/cell. At the indicated times post-infection, the cells were treated with 100 μg/ml cycloheximide to arrest ribosome transit. Cells were collected from the dishes with a 1X trypsin solution containing 100 μg/ml cycloheximide. Cells were swollen in low salt buffer (20 mM Tris-HCl pH 7.4, 10 mM NaCl and 3 mM $MgCl_2$), and then lysed with low salt buffer containing 1.2% Triton N-101 and 200 mM sucrose,

and 9 strokes of a Dounce homogenizer. Cell nuclei were removed by brief centrifugation, and cell lysates were layered onto 0.5 M – 1.5 M sucrose gradients prepared in low salt buffer. The gradients were centrifuged for 58 minutes at 159,000 x *g* in a Beckman SW55 rotor and fractionated with an ISCO density gradient fractionator with an absorbance monitor at 254 nm. RNA was extracted from 500 µl fractions with 1:1 phenol/chloroform, then with chloroform, and finally precipitated with 250 mM NaCl, 20 µg/ml glycogen, and ethanol. The RNA from each fraction was electrophoresed through 1.2% agarose/6% formaldehyde gels and subjected to Northern hybridization analysis as described above.

# CHAPTER THREE

## GENE 5 AND 6 MODEL TRANSLATIONAL EFFICIENCIES OF ROTAVIRUS mRNA

### Introduction

The rotavirus genome consists of 11 segments of double stranded RNA (dsRNA) that are packaged into a structurally complex triple-layered particle [271]. Viral mRNAs transcribed from dsRNA have a 5' terminal cap structure $m^7GpppG^{(m)}Gpy$ and are not polyadenylated [118, 119]. Nine of the 11 dsRNA segments are monocistronic, the exceptions being gene 11 that encodes NSP5 and NSP6, and the VP7 gene that has an alternative initiation codon [115, 117]. The open reading frame of each gene is flanked by 5' and 3' untranslated regions (UTR) that vary in length and sequence between each segment, although short consensus sequences at both the 5' and 3' ends are conserved [145]. Rotavirus mRNAs transcribed within subviral particles are extruded into the cytoplasm through pores in the capsid layers located at the icosahedral 5-fold axes [272]. The mRNAs are translated into protein or transported to sites of virus assembly where they are packaged as ssRNA, and then replicated into dsRNA subsequent to packaging.

The molecular mechanisms that regulate transcription and subsequent translation of individual rotavirus genes are not well defined. Johnson and McCrae measured the levels of viral transcripts and encoded proteins and showed that there was quantitative as well as temporal control of rotavirus gene expression [29]. The 11 viral proteins were

not synthesized to equivalent levels in infected cells, nor did the levels of individual proteins correspond with the levels of the cognate mRNAs. These observations suggested both transcriptional and translational control of viral gene expression. Further, these data implied that signals in some, if not all, of the 11 viral mRNAs regulated the levels of specific viral proteins. The mechanisms of regulation were not defined.

Few studies have investigated the role that protein synthesis plays in controlling expression of rotavirus genes throughout the replication cycle. A recent study identified a specific interaction between nonstructural protein NSP3 and the cellular translation initiation factor eIF4GI [249]. NSP3 is a sequence-specific RNA binding protein that binds to the last 4-5 conserved nucleotides of the 3'end of viral mRNAs [247]. eIF4GI is a component of the eIF4F initiation complex that recognizes the 5' cap structure typical of eukaryotic mRNAs. The interaction between NSP3 and eIF4GI likely promotes circularization of viral mRNAs to enhance translation, as previously proposed [248, 249]. At the RNA level, a four-nucleotide translation enhancer element in the 3' terminal consensus sequence of rotavirus mRNAs has been reported [240]. Together, these studies provide initial evidence that *trans*-acting viral proteins and *cis*-acting signals in viral mRNA contribute to controlling synthesis of rotavirus proteins in infected cells.

We investigated potential mechanisms of post-transcriptional regulation of rotavirus gene expression by analyzing the translational efficiencies of gene 5 and gene 6 mRNA. These two genes were chosen as models for analysis of rotavirus gene

regulation because 1) both are expressed early in infection, 2) VP6 is expressed to higher levels than NSP1, and 3) the mRNAs and ORFs are close in size, thus minimizing potential variations in expression due to mRNA length. Gene 5 encodes nonstructural protein NSP1. The function of NSP1 in infected cells is not clear, but NSP1 appears to be nonessential for replication because viruses with gene 5 rearrangements that do not synthesize NSP1 have been isolated [260, 273-276]. Gene 6 encodes the major inner capsid protein VP6, which is the subgroup specific antigen and is required for viral transcriptase activity [117]. We examined mRNA accumulation, protein stability, and polyribosome distribution of genes 5 and 6 mRNA in rotavirus-infected cells. Our data suggest the difference in the amounts of NSP1 and VP6 is accounted for by a difference in the translational efficiencies of the mRNA.

## Results

### Synthesis of NSP1 and VP6 in B641-Infected Cells

NSP1 and structural protein VP6 are expressed early in infection [252]. Johnson and McCrae reported that in BSC-1 cells infected with bovine rotavirus strain UK (Compton), VP6 was present in approximately 25-fold molar excess over NSP1 [29]. We analyzed the relative amounts of NSP1 and VP6 in B641-infected MA104 cells to establish the levels of accumulation of these two proteins in our virus-cell combination. Cells were mock infected or infected with B641 in the presence of TRAN-$^{35}$S label. The cultures were harvested 4.5 hours post-infection and labeled proteins were separated by SDS-PAGE and visualized by autoradiography. Figure 3.1 shows the typical pattern of

rotavirus protein synthesis. Quantification of ~50 kD NSP1 and ~44 kD VP6 by densitometry showed VP6 present in at least 10-fold excess over NSP1. This is likely an underestimation of the magnitude of the difference between the two proteins, since NSP1 has approximately 2.5 times the number of methionines and cysteines present in

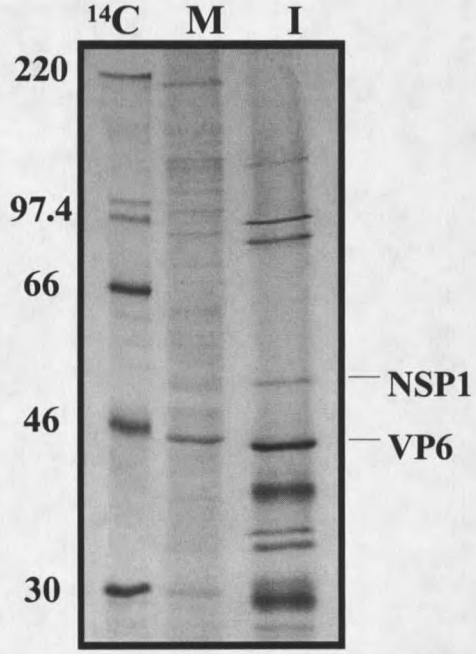



Figure 3.1: Metabolic labeling of rotavirus B641 proteins. MA104 cells in 60 mm dishes were mock-infected (M) or infected with B641 (I) in the presence of TRAN$^{35}$S-label and 10 μg actinomycin D ml$^{-1}$. Cultures were harvested 4 h p.i. in RIPA buffer. Proteins were separated by 10% SDS-PAGE and visualized by autoradiography. Proteins were quantified with a Bio-Rad Molecular Imager FX with Quantity One software

VP6. Analysis of the levels of VP6 and NSP1 at 6 and 8 hours post-infection indicated the relative ratios of these two proteins at later times in the infection remained at 10-fold (6 hr) or increased to 15-fold (8 hrs) (data not shown). These data suggested differential regulation of synthesis of VP6 and NSP1 was maintained throughout the

infectious cycle. A pulse-chase analysis was performed to determine if differences in protein stability accounted for the excess of VP6 over NSP1. Pulse-labeling at four hours post-infection followed by chase times up to three hours indicated no significant difference in the stability of NSP1 and VP6, with both proteins displaying half-lives of approximately 45 minutes (Figure 3.2). These data are in agreement with those reported by Hundley and co-workers, [277] and suggest expression of these two proteins must be regulated at the RNA level.

mRNA Levels for Genes 5 and 6 Four Hours Post-Infection are Similar

Synthesis of NSP1 and VP6 may be transcriptionally controlled. If this is the case, to account for such a difference in the relative amounts of each protein, the levels of the encoding mRNAs should differ dramatically. Accumulation of the mRNA for genes 5 and 6 was measured to determine if synthesis of NSP1 and VP6 was transcriptionally regulated. Trypsin-activated B641 was adsorbed to MA104 cells at 4°C to synchronize the infection. Rotavirus adsorbs to cells at this temperature but does not penetrate the cell membrane and initiate the replication cycle [262]. After the adsorption period, the infected cultures were shifted to 37°C to allow internalization and continuation of the replication cycle. Total RNA from infected cultures was harvested every hour for four hours, and accumulation of mRNA for genes 5 and 6 was analyzed by Northern hybridization to gene-specific probes that were comparable in length and specific activity. The data in Figure 3.3 show the levels of each mRNA up to and including four hours post-infection were not significantly different. Quantification of the radioactive signals indicated the magnitude of the difference between the two

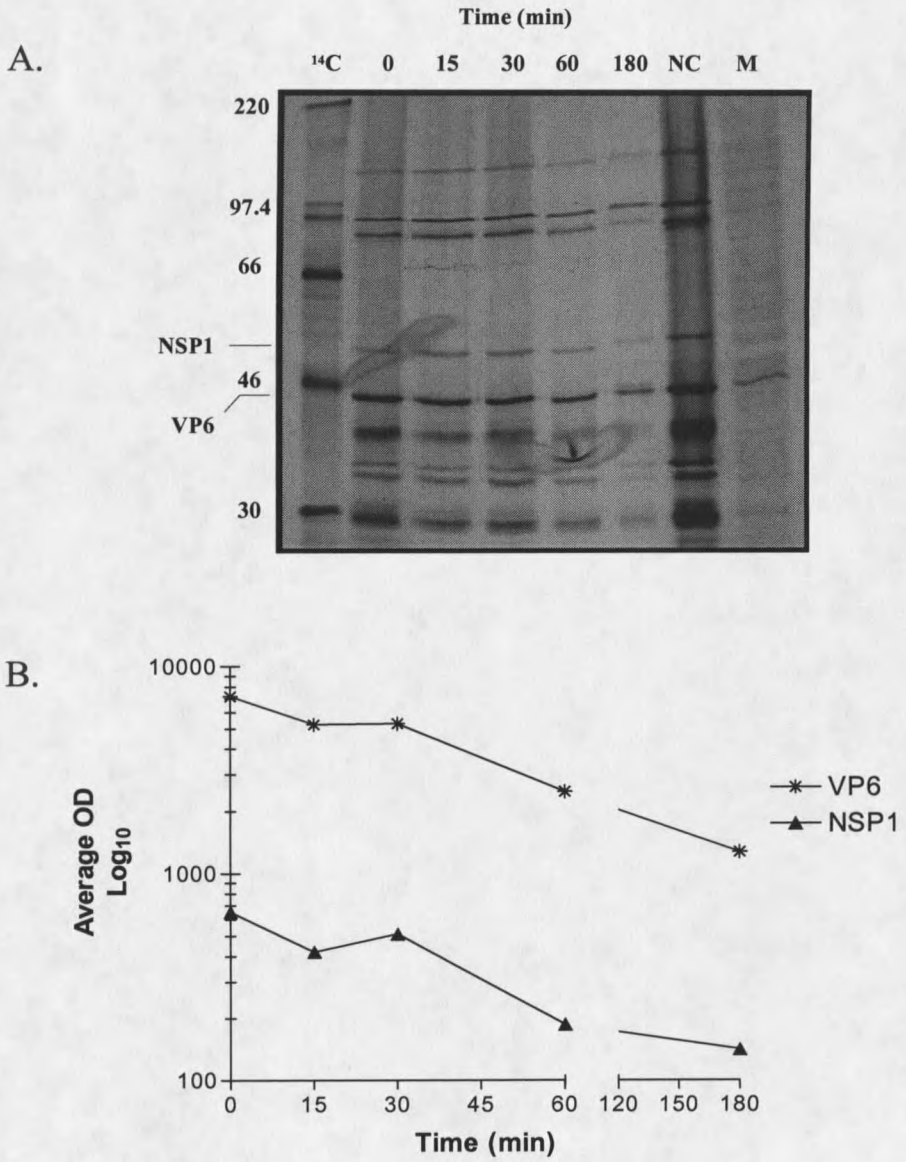
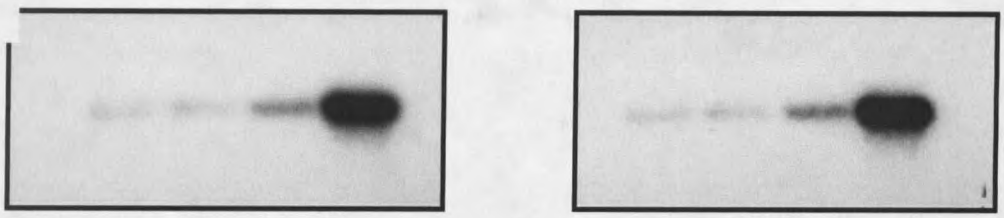
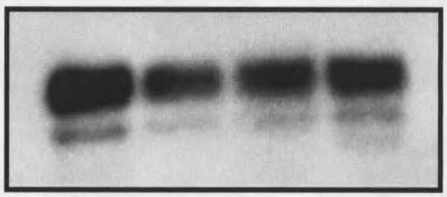



Figure 3.2: Pulse-chase labeling of B641 proteins MA104 cells in 60 mm dishes were infected with B641 at an m.o.i. of 10. At 4 h p.i., infected cells were pulsed for 30 min with 40 μCi TRAN$^{35}$S-label ml$^{-1}$, then chased for indicated times with medium containing 400X methionine and cysteine and 100 μg cycloheximide ml$^{-1}$. Cultures were harvested in RIPA buffer and labeled proteins were separated by 10% SDS-PAGE. (A) Autoradiograph of pulse-chase analysis. M, mock-infected; NC, no chase. (B) Relative amounts of NSP1 and VP6 and decay rates quantified with a Bio-Rad Molecular Imager FX and Quantity One software and plotted as a decrease in signal over time.

A.



Gene 5 Gene6



Actin

B.

| Time (hr) | Gene 5 (Avg OD) | | Gene 6 (Avg OD) | Actin (Avg OD) |
|---|---|---|---|---|
| 1 | 182.19 | 1.4X | 130.59 | 5138.82 |
| 2 | 175.84 | 1.24X | 141.73 | 2955.80 |
| 3 | 256.69 | 1.13X | 227.29 | 3250.28 |
| 4 | 2396.53 | 1.13X | 2124.12 | 3589.49 |

Figure 3.3: Accumulation of gene 5 and gene 6 mRNA. MA104 cells were infected with B641. RNA was extracted from parallel cultures with Trizol® reagent every hour for 4 hours. The RNA was electrophoresed on 1.2% agarose/formaldehyde gels, transferred to nylon membranes and hybridized with $^{32}$P-labelled probes specific for gene 5, gene 6, or actin as a loading control (A). Signals were quantified with a Bio-Rad Molecular Imager FX with Quantity One software (B).

mRNAs was less than 2-fold. These data, taken together with the protein analyses described above, suggest another mechanism of regulation must account for the differences in the amounts of NSP1 and VP6. Thus we asked if the low amount of NSP1 synthesized early in infection was due to an inefficiently translated gene 5 mRNA.

Distribution of Gene 5 mRNA in Polyribosome Gradients

Analysis of polyribosome distributions can determine whether an mRNA is efficiently translated, and if the efficiency of translation is regulated at the initiation step [270]. We first evaluated the distribution of gene 5 mRNA in polysome gradients to determine if the low expression of NSP1 compared to VP6 was due to differences in the translational efficiencies of the two mRNAs. B641-infected MA104 cells were harvested four hours post-infection and the lysate was centrifuged through 0.5-1.5 M linear sucrose gradients. RNA was extracted from each fraction and the distribution of gene 5 mRNA in the gradients was analyzed by Northern hybridization. Figure 3.4 shows the polyribosome sedimentation profile of the infected cell lysate (Figure 3.4A) and the sedimentation of gene 5 mRNA in the gradient (Figure 3.4B). Gene 5 mRNA displayed a prominent bimodal distribution, with RNA detected in polysome fractions (fractions 5-11), and subpolysomal fractions (1-4) containing ribosomal subunits, untranslated messenger ribonucleoprotein particles, and pre-initiation complexes. Approximately 55% of gene 5 mRNA was found in subpolysomal fractions. We analyzed the distribution of gene 6 mRNA for comparison and control (see Figure 3.6, left panel). In contrast to gene 5 mRNA, the majority of gene 6 mRNA was associated

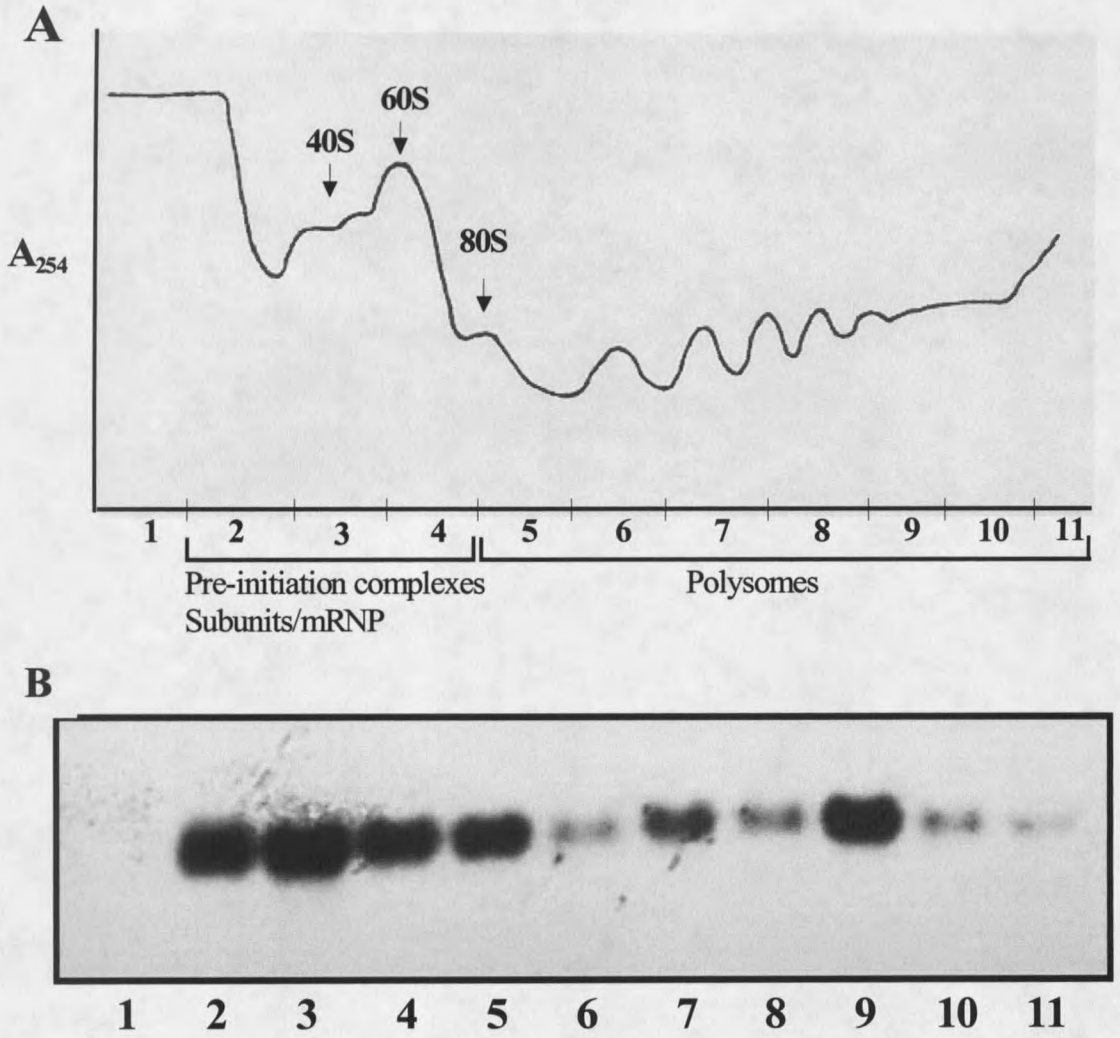



Figure 3.4: Bimodal distribution of gene 5 mRNA in polyribosome gradients. B641-infected cell lysates harvested 4 h p.i. were centrifuged for 58 min through 0.5-1.5 M sucrose gradients at 150,000 x*g* in an SW55 Beckman rotor. The gradients were fractionated with an ISCO density gradient fractionator with an absorbance monitor at 254 nm. The sedimentation profile is shown in (A). (B) RNA was extracted from each fraction, separated on a 1.2% agarose/formaldehyde gel and transferred to nylon membrane. The blot was probed with a $^{32}$P-labelled gene 5-specific probe. Radioactive signals were quantified with a Bio-Rad Molecular Imager FX with Quantity One software. Lane numbers correspond to gradient fractions.

with large polysomes. Quantification of the hybridization signal indicated ~72% of gene 6 mRNA was detected in polysomal fractions. The observed distribution of gene 6 was anticipated based on the high levels of VP6 detected four hours post-infection. The rotavirus replication cycle in monkey kidney cells in culture is approximately eight hours [29]. We performed additional polysome analyses of both genes as described above at 2 and 8 hours post-infection to determine if the observed sedimentation was altered at earlier and later times (Figure 3.5). At 2 hours post-infection, ~67% of gene 5 mRNA and 52% of gene 6 (data not shown) was present in subpolysomal fractions (Figure 3.5A). At 8 hours post-infection, ~89% of the gene 5 mRNA, and 69% of gene 6 mRNA (data not shown) was subpolysomal (Figure 3.5B). This predominately subpolysomal sedimentation of gene 5 and 6 mRNAs at 8 hours post-infection may represent mRNA present in subviral particles or replication intermediates. Significant cytopathic effect is observed at this time, evidenced by a decrease in the amplitude of the absorbance profile. These data clearly indicated that half or more of transcribed gene 5 mRNA was not being translated throughout the course of the replication cycle. Taken together, these data suggested that there was a difference in the translational efficiency of gene 5 and gene 6 mRNA, and the translational inefficiency of gene 5 was maintained throughout the replication cycle.

NSP1 Expression is Regulated at Initiation of Translation

The distribution of gene 5 mRNA in Figures 3.4 and 3.5 reflects an inefficient initiation of translation, as more than half of the mRNA is not polysome-associated. An alternative interpretation of this distribution is that the significant proportion of gene 5

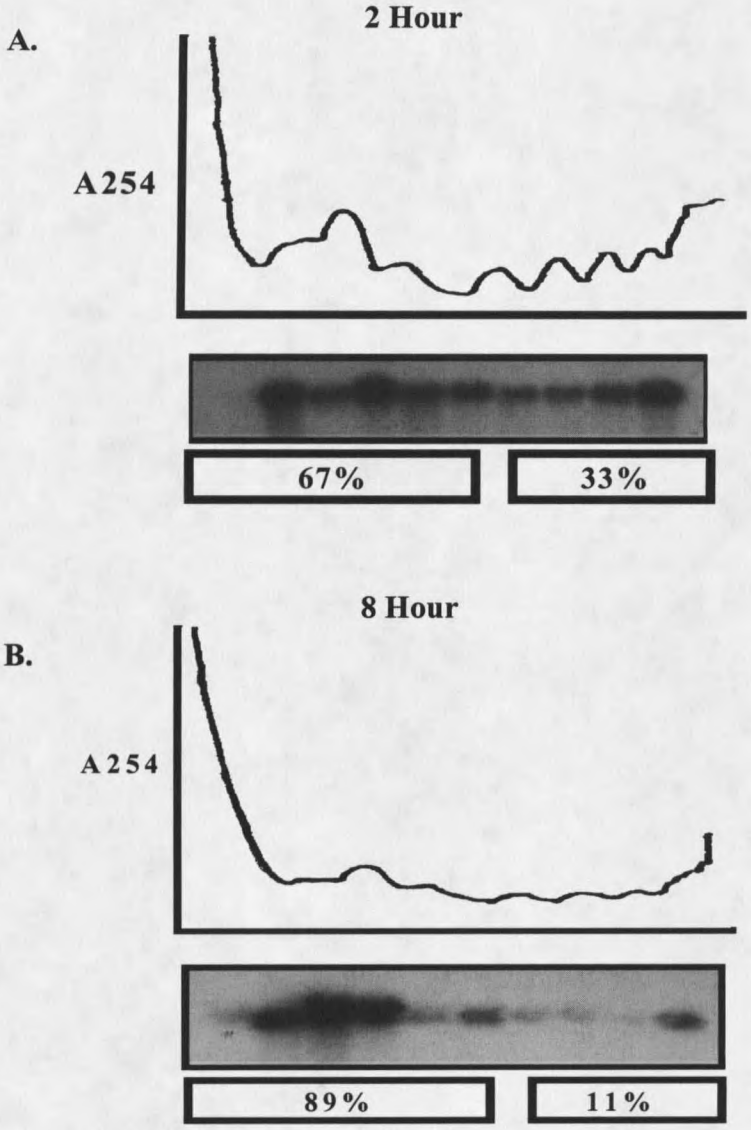



Figure 3.5: Distribution of gene 5 mRNA in polyribosme gradients at 2 and 8 h p.i. B641-infected cells were treated with 100 μg/ml cycloheximide $ml^{-1}$ for 45 min prior to harvest at 2 h p.i. (A) and 8 h p.i. (B). Lysates were centrifuged through a 0.5-1.5 M linear sucrose gradients for 58 min at 159,000 x *g* in an SW55 rotor. The top panels are absorbance profiles. The lower panels show Northern blot hybridizations of gene 5 mRNA extracted from gradient fractions. Radioactive signals were quantified with a Bio-Rad Molecular Imager FX with Quantity One software. Percentages of mRNA in polysomal and subpolysomal fractions are shown below the blots. Data shown are representative of multiple experiments.

mRNA in subpolysomal fractions of the gradient may be untranslatable and bound in messenger ribonucleoprotein particles (mRNP). The presence of rotavirus mRNA in untranslated mRNP is a formal possibility, given that viral mRNAs must also serve as plus-strand templates for packaging and replication into dsRNA. To distinguish between these two interpretations of the data, infected cells for polysome analysis were treated with 0.5 μg/ml cycloheximide for 45 minutes prior to harvest. Low concentrations of cycloheximide (compare 0.5 μg/ml in this experiment to 100 μg/ml to arrest ribosome transit in the previous experiment) can effectively mobilize inefficiently translated mRNAs onto polyribosomes by slowing the elongation rate relative to the initiation rate [270, 278, 279]. The result then, is an increase in the number of ribosomes per mRNA and a consequent shift in the mRNA from lighter to heavier fractions of a sucrose gradient. If the low level of NSP1 expressed in infected cells is a result of inefficient initiation of translation on gene 5 mRNA, then such a shift in distribution of the mRNA in the gradient should be observed when infected cells are treated with low doses of cycloheximide. The data presented in Figure 3.6 suggest gene 5 mRNA is a poor initiator of translation. The distinctive bimodal distribution of gene 5 in infected cells without cycloheximide was similar to that of the experiment shown in Figure 3.4, as ~42% of the mRNA displayed subpolysomal sedimentation and ~58% of the mRNA was found associated with polysomes (Figure 3.6, left panel). Treatment of infected cells with 0.5 μg/ml of cycloheximide resulted in a shift in the distribution of the mRNA toward the heavier fractions of the gradient containing large polysomes (Figure 3.6, right panel). Quantification of the hybridization signals indicated that

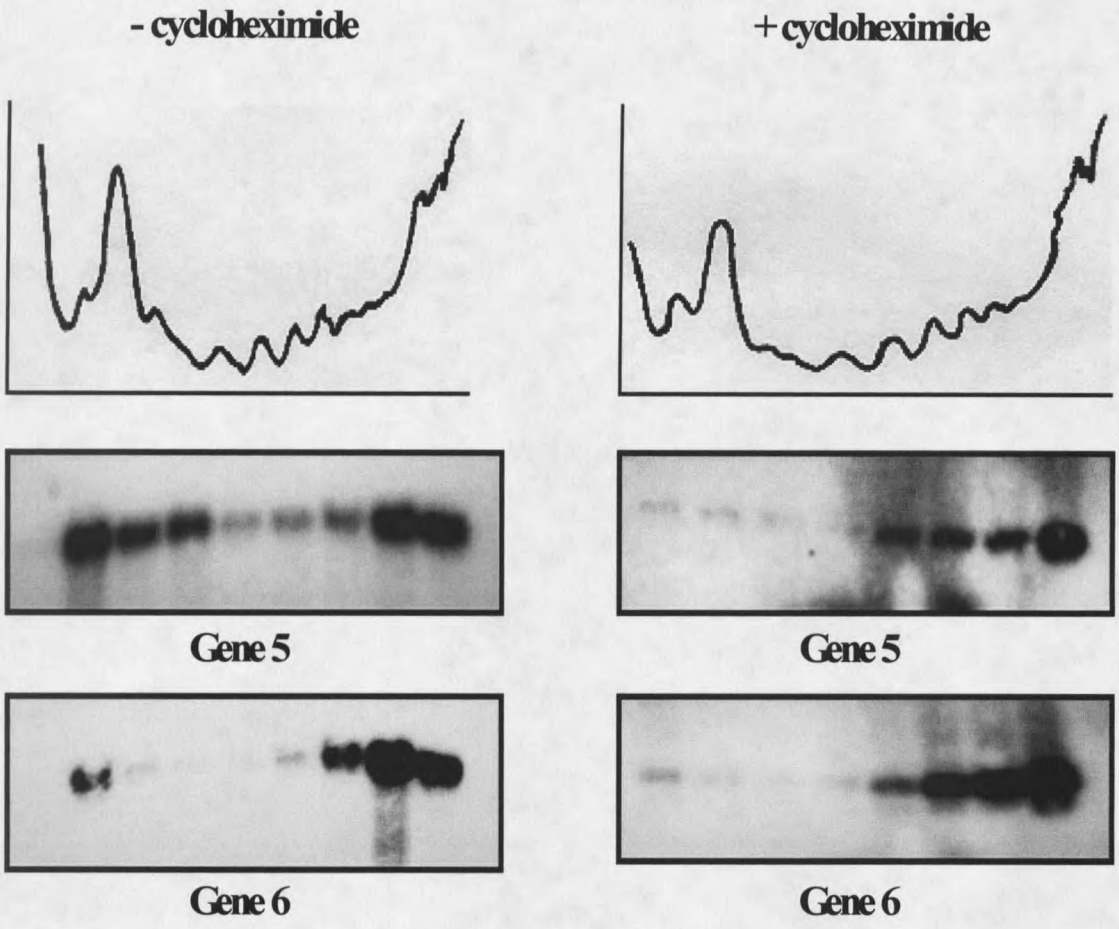



Figure 3.6: Distribution of gene 5 and gene 6 mRNA in the presence of low doses of cycloheximide. B641-infected cells were treated with 0.5 μg cycloheximide $ml^{-1}$ for 45 min prior to harvest. Lysates were centrifuged through 0.5-1.5 M linear sucrose gradients for 58 min at 159,000 x *g* in an SW55 rotor. The top panels are absorbance profiles in the absence (left panels) or in the presence (right panels) of cycloheximide. The lower panels show Northern blot hybridization of genes 5 and 6 in the absence (left) or presence (right) of cycloheximide. Radioactive signals were quantified with a Bio-Rad Molecular Imager FX with Quantity One software. Data shown are representative of multipe experiments.

~80% of gene 5 mRNA was found in polysome fractions in the presence of cycloheximide. Northern hybridization to detect gene 6 mRNA also demonstrated a detectable shift (~72% to ~93%), but not nearly as dramatic as observed for gene 5, in that the amount of gene 6 mRNA in the lighter fractions never approached that observed for the gene 5 mRNA. The ability of low concentrations of cycloheximide to mobilize a substantial amount of gene 5 mRNA onto polyribosomes suggests that under native infected cell conditions, gene 5 is an inefficiently translated mRNA.

## Discussion

We investigated mechanisms of rotavirus gene regulation by analyzing mRNA accumulation, protein stability, and polyribosome distributions of two differentially expressed genes encoding NSP1 and VP6. The data show that the mRNA encoding NSP1 is inefficiently translated. A bimodal sedimentation and the response of gene 5 mRNA to low concentrations of cycloheximide indicate expression of NSP1 is translationally controlled at the initiation step of protein synthesis. Analysis of several time points showed the sedimentation of gene 5 mRNA remained bimodal (2, 4, and 6 hours) or predominately subpolysomal (8 hours), suggesting regulation of NSP1 expression was maintained throughout the replication cycle. This distribution of gene 5 mRNA at the 8 hour time point may correlate with a switch from viral protein synthesis to packaging of the mRNA template and replication into dsRNA. The sedimentation of gene 6 mRNA in polysome gradients supports the conclusion that gene 5 mRNA is

inefficiently translated by providing evidence that a prominent subpolysomal sedimentation is not an inherent property of rotavirus mRNA.

NSP1 is the least conserved protein in the rotavirus genome, and its function in the replication cycle is not known. The cysteine-rich N-terminal domain of NSP1 contains a putative zinc-finger motif similar to those in eukaryotic transcription factor TFIIIA, and this region is required to bind the 11 viral mRNAs in vitro [238, 253]. The low level of expression of NSP1 persists throughout the replication cycle [252]. The number of functional molecules of NSP1 in infected cells is likely tightly controlled, consistent with its role as a regulatory protein. Interestingly, NSP1 appears to be dispensable for virus replication, as mutant rotavirus strains with rearrangements in gene segment 5 that do not encode NSP1 are viable in cell culture [260, 274, 276, 277, 280]. Isolation of such mutants provides further support for the role of NSP1 as a regulatory protein, in that viral strains defective for NSP1 expression display a small plaque phenotype compared to their wildtype counterparts.

In contrast to a regulatory function for NSP1, VP6 forms the major inner capsid layer of the mature virus particle. 260 trimers of VP6 are incorporated into a mature virion [271]. If a single infected cell releases 50-60 pfu [29], then the number of molecules of VP6 that must be synthesized falls between 39,000 and 46,800. In fact, this number is probably much higher, given that this calculation assumes a particle to pfu ratio of 1, and the particle to pfu ratio for rotavirus has been reported as high as 100:1. In either case, VP6 must be synthesized in high amounts, and the mRNA encoding VP6 must be translated more efficiently than that encoding NSP1.

*Cis*-acting signals in rotavirus mRNAs and *trans*-acting viral proteins that function in regulating rotavirus gene expression have only recently come under study. Rotavirus belongs to the family *Reoviridae*, and studies of translation of reovirus mRNA provided a significant amount of original data on the selectivity of the cellular translational apparatus, and the features of a viral mRNA that might contribute to such selectivity [189, 267, 278, 281, 282]. Walden and co-workers reported hierarchal translation efficiency of mRNAs transcribed from reovirus gene segments [278]. A series of studies led this group to propose that translation rates in reovirus-infected cells are regulated by competition of host and viral mRNAs for limiting translation factors, and the competitive ability of a mRNA was determined by its ability to efficiently recruit translation initiation factors [278, 283-285]. Direct competition between rotavirus gene 5 and gene 6 mRNA was not addressed in this study.

Structural features in the 5' UTR of a mRNA that can dictate its translation efficiency are well established and include cap-proximal secondary structure and length of the UTR [286-288]. The 5' UTR of gene 5 of B641 is 31 bases, longer than the 23 nucleotide 5' UTR of gene 6. Additional ribonucleotides in the gene 5 5'UTR might contribute to a secondary structure that is inhibitory to translation initiation. However, the 5'UTR of the 11 rotavirus gene segments range from 9 to 49 nucleotides, and the lengths of the UTRs do not correlate with the level of the encoded protein. For example, VP3 is a structural core protein synthesized to low, but detectable levels in infected cells and has a 49 base 5' UTR. Though these observations do not dismiss a role for the 5' UTR in translational efficiencies of rotavirus mRNA, there must be

additional components of the regulatory mechanism. Such mechanisms include a potential interactive synergy with the 3' UTR and differing affinities of RNA binding proteins, viral or cellular, for 5' and 3' ends of each viral mRNA. We also noted a short non-overlapping upstream ORF immediately preceding the start codon in gene 5 of B641, and of bovine strains RF [265], UK [253], and A44 [289], that could negatively affect the efficiency of translation of this mRNA. Translational control by upstream ORFs has been documented for a number of viral and cellular mRNAs, and in some cases, reduces expression of the downstream ORF [290].

Evidence continues to accumulate that implicates the 3'UTR in translational control of gene expression in both cellular and viral mRNA [242, 291-295]. Tanguay and Gallie reported that a longer 3'UTR on a mRNA lacking a polyadenylate tail increased the translational efficiency of a luciferase reporter mRNA, and this effect was sequence independent and gene independent [242]. Rotavirus mRNAs do not have polyA tails, and unlike the comparison between the 5'UTR of genes 5 and 6, the 3'UTRs are quite different. The 3'UTR of gene 5 is 50 bases long compared to 139 bases for gene 6. The length of the 3'UTR may play a more important role in regulating translation efficiency of rotavirus mRNA than the 5'UTR. A mechanism for how the length of a 3'UTR enhances the translation efficiency of an mRNA has been proposed [242]. It was suggested that following termination and dissociation of the 80S ribosome, the 40S ribosomal subunit continues to transit the 3'UTR. Therefore, mRNA with a longer 3'UTR would have a higher local concentration of ribosomal subunits available to reinitiate translation of the same mRNA. This seems a plausible

explanation for the difference in translational efficiencies of genes 5 and 6, certainly under conditions of competition for limiting translation factors early in the rotavirus replication cycle.

A recent report identified a conserved four nucleotide enhancer sequence present at the 3'termini (GACC) of rotavirus genes that enhanced expression of a luciferase reporter gene when RNA encoding this sequence was transfected into rotavirus infected cells [240]. These four nucleotides, when present at the 3' terminus of rotavirus mRNA, bind NSP3 [241], and it was proposed that variation in this sequence could negatively affect viral protein expression by altering binding of NSP3 [296]. The data reported thus far were gained from analysis of luciferase reporter genes, and whether such variation in the 3'terminal sequence causes a decrease in synthesis of the native protein in the context of an infection remains to be determined. Regardless, these data suggest an intriguing role for the 3' UTR in regulation of protein synthesis programmed from viral mRNA in rotavirus infected cells. Future studies will address the role of sequences and structures of rotavirus UTRs in regulating viral protein synthesis, and *trans*-acting factors that function in regulating expression levels of rotavirus genes throughout the replication cycle.

# CHAPTER FOUR

# THE ROLE OF NSP1 IN REGULATION OF ROTAVIRUS GENE EXPRESSION

## Introduction

During rotavirus infection, the viral mRNA are produced in nonequimolar amounts and the levels of the viral proteins do not correspond with the levels of cognate mRNAs [29]. This suggests that there is regulation of gene expression at both the level of transcription and at the level of translation. We have shown that the differences in the protein levels are due in part to a difference in the translation efficiencies of the mRNAs [297]. Gene 6 mRNA, encoding VP6, is translated more efficiently that gene 5 mRNA, encoding NSP1. Further studies showed that gene 5 mRNA is a comparatively poor template for translation initiation. The mechanisms for this regulation and the proteins involved are not well understood.

Transcriptionally active double-layered rotavirus particles synthesize viral mRNAs that are capped, but not polyadenylated [118]. The 5' and 3' untranslated region (UTR) vary in length, but contain two consensus sequences that are present in all viral mRNAs [25, 118]. The 3' consensus sequence of group A rotaviruses is 5'((A/U)U(U/G)UGACC)3', with the last 5 nucleotides (UGACC) being highly conserved among the 11 segments [118, 119]. Poncet and coworkers reported that NSP3 binds to the 3' end of the viral transcripts in a serogroup-dependent manner, and

that the binding of recombinant NSP3 to the mRNA requires the last 5 bases [241, 247]. Both the monomeric and homodimeric form of NSP3 bind to the 3' end of rotavirus mRNA [241].

Most cellular mRNA contain a 5' cap structure ($m^7$GppN) and a 3' polyadenylated (polyA) tail [199, 200]. Together, these two structures enhance the initiation of translation of the host cell mRNA [203-209]. Studies have shown that the synergistic effect of the cap structure and the poly(A) tail is mediated through the poly(A)-binding protein (PABP). PABP not only interacts with the 3' poly(A) tail, but also with eIF4G [203, 210, 211]. This interaction causes the mRNA to become circularized and is thought to increase the translation efficiency of the mRNA by continuous recycling of the ribosomes for reinitiation of translation [212].

Currently, NSP3 is the only viral protein known to participate in the translation of the viral mRNA by seizing the eukaryotic translation machinery. NSP3 interacts with eIF4GI [249], and the simultaneous interaction of NSP3 with the 3' end of viral mRNA and eIF4GI is needed for efficient translation of the viral mRNA [248]. This suggests that NSP3 plays a functional role analogous to PABP and causes circularization of the viral mRNA. Furthermore, eIF4G has a higher affinity for NSP3 than it does for PABP, and NSP3 is able to outcompete PABP and consequently enhance translation of viral mRNA over the host cell mRNA [210, 249].

We hypothesize that NSP1 also plays a regulatory role in translation of viral mRNA. NSP1 is a 53 kDa protein that is expressed at low levels during the replication cycle [29]. NSP1 was shown to interact with NSP3 in the yeast two-hybrid system, but

this interaction has yet to be shown in infected cells [254]. NSP1 binds the 11 viral mRNA in vitro [245]. The NSP1-RNA-binding site on the transcripts is not known [245].

The affinity of NSP3 for the different viral mRNA has not been experimentally examined, yet it was suggested that NSP3 binds to the 11 transcripts with the same affinity, because the NSP3 homodimer recognizes the last four 3' consensus nucleotides [241, 246, 247]. Therefore, this suggests other proteins or signals within the mRNA are important in regulating translation of rotavirus mRNAs. To investigate the role of NSP1 in the regulation of viral gene expression, the distributions of gene 6 and gene 11 in polysome gradients were compared between a mutant strain (A5-16) and a wildtype strain (NCDV) of rotavirus. A5-16 is a rotavirus strain isolated from diarrheic calf [260]. Gene 5 of A5-16 has a large deletion of 500 bases that includes the cysteine-rich zinc finger motif. Therefore, if a protein is synthesized from gene 5 mRNA, the protein does not have the capability of binding to the viral mRNA. This deletion, also results in a nonsense mutation at nucleotides 183-185 that creates a termination codon that would produce a protein of only 50 amino acids [260]. Since two different bovine rotavirus strains are being used in this study, gene 6 and gene 11 were chosen to be analyzed due to the high sequence similarity between strains [115, 298]. The studies in this chapter suggest NSP1 plays a regulatory role in the translation of viral mRNAs.

## Results

### A5-16 and NCDV Patterns of Viral Protein Synthesis

The A5-16 bovine rotavirus strain was previously shown not to express NSP1 [260]. We verified that our virus preparation replicated stably and resulted in the same protein profile as the originally isolated clone. MA104 cells were mock infected or infected with either NCDV or A5-16. Viral proteins were labeled with TRAN$^{35}$S-label and the cells were harvested at 4 hours post-infection. Similar amounts of total protein were analyzed by SDS-PAGE and the separated proteins were visualized by autoradiography. Figure 4.1 shows that at fours post infection, a band of approximately

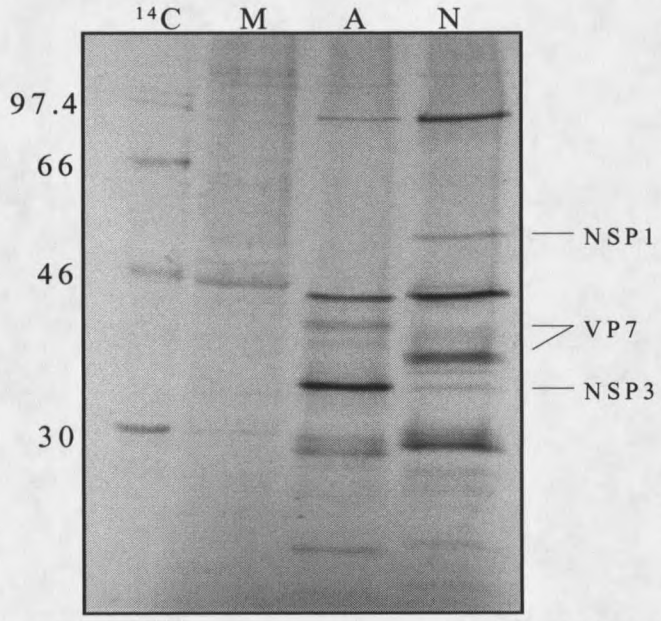



Figure 4.1: Metabolic labeling of rotavirus A5-16 and NCDV protein synthesis. MA104 cells in 35 mm dishes were mock-infected (M) or infected with A5-16 (A) or NCDV (N) in the presence of TRAN35S-label and 10 μg actinomycin D ml-1. Cultures were harvested 4 h p.i. in RIPA bufer. Proteins were seperated by 10% SDS-PAGE and visualized by autoradiography. Rotavirus proteins are indicated. The difference in the migration of VP7 between the two strains is due to differences in the glycosylation.

50 kDa corresponding to NSP1 was present in the NCDV infected cell lysates, but not in the cells infected with A5-16. Taniguchi and co-workers previously reported that the A5-16 gene segment 5 mRNA was unable to produce NSP1 in vitro in a rabbit reticulocyte system, or in vivo. Long exposure times, gels of short runs, and immunoprecipitations were unable to produce a protein of any size encoded by gene 5 of the mutant strain [260].

The band corresponding to NSP3 was much stronger in the A5-16 infection than in the NCDV infection. As previously discussed by Taniguchi and co-workers, the strong band represented by NSP3 in the A5-16 strain was not due to gene 5 producing a product of similar molecular weight [260]. Figure 4.1 also shows a difference in the migration of VP7. The difference in migration is due to the difference in the VP7 glycosylation between the two strains. Two glycosylation sites are present in VP7 of the A5-16 strain and one is present for the NCDV strain [299]. These data verified that our virus preparation resulted in the same protein profile originally reported for A5-16.

Differential Regulation Among the Rotavirus mRNAs

We analyzed the distribution of gene 6 from A5-16 and NCDV infected cells in polyribosome gradients to determine if there was a difference in the translation efficiencies of gene 6 mRNAs between the two strains. MA104 cells were infected with either A5-16 or NCDV and the cells were harvested at two hours post infection. The lysates were layered on 0.5-1.5 M linear sucrose gradients and analyzed by Northern blot hybridization. Figure 4.2 shows the sedimentation of gene 6 mRNA for

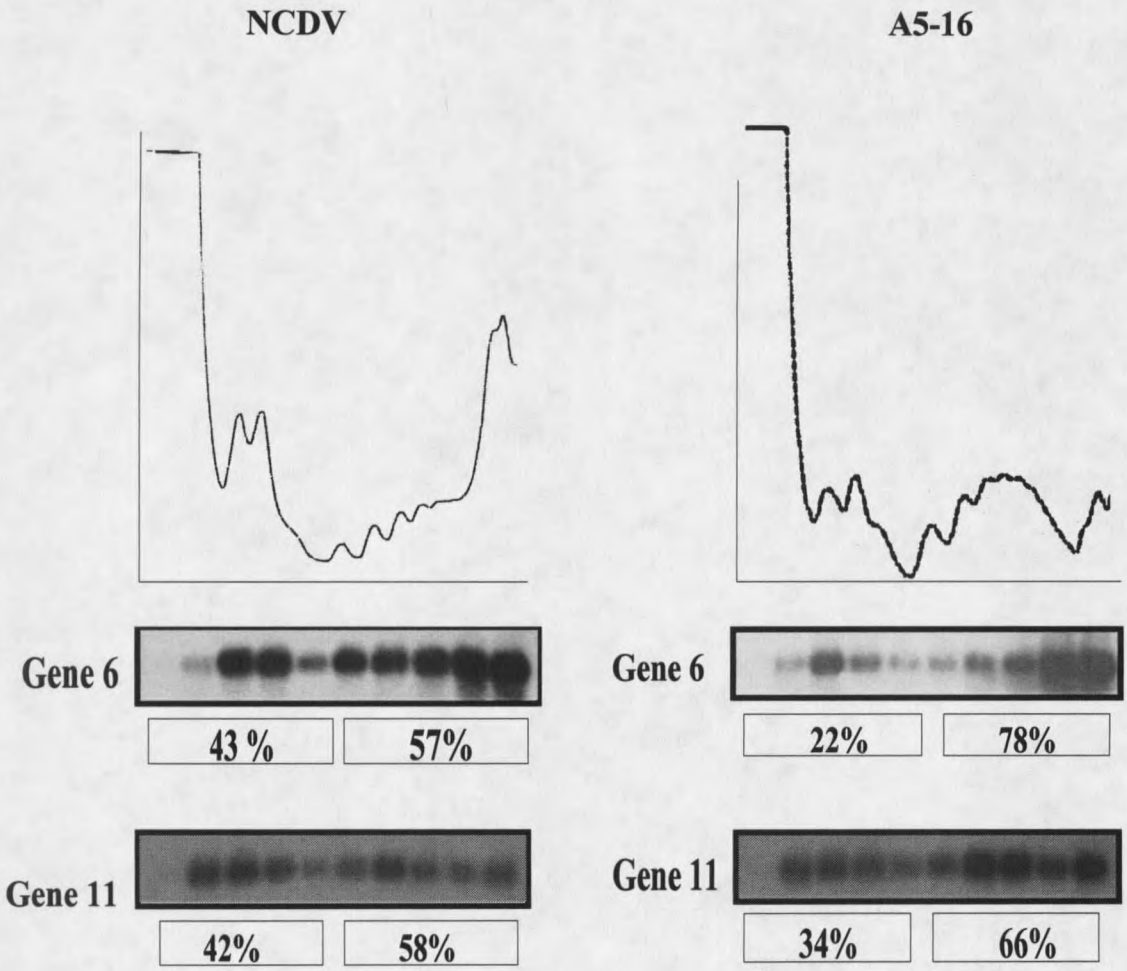



Figure 4.2: Distribution of genes 6 and 11 mRNA in polyribosome gradients 2 hours post infection. NCDV-infected lysates (left panel) or A5-16-infected lysates (right panel) harvested at 2 h p.i. were centrifuged through 0.5-1.5 M linear sucrose gradients for 58 minutes at 159,000 x *g* in a SW55 rotor. The top panels are absorbance profiles. The lower panels show Northern blot hybridization of genes 6 and 11. Radioactive signals were quantified with Bio-Rad Molecular Imager FX with Quantity One software. Percentages of mRNA in polysomal fractions are shown below blots.

NCDV-infected and A5-16-infected cells. Approximately 57% of NCDV gene 6 mRNA was located thepolysomal fractions. In contrast, a large majority of A5-16 gene 6 mRNA was found in the polysomal fractions (78%). These data showed a difference in the sedimentation of NCDV gene 6 mRNA and A5-16 gene 6 mRNA, suggesting a difference in the regulation of gene specific translation efficiencies of gene 6 mRNA between the two viral strains at two hours post infection.

Gene 11 mRNA was analyzed for comparison to determine if the differences found in the sedimentation of gene 6 mRNA between the two strains occurred for all rotavirus mRNA. Figure 4.2 also shows the sedimentation profile of gene 11 mRNA for A5-16 and NCDV-infected cells at two hours post infection. In NCDV-infected cells approximately 42% of gene 11 mRNA was found in the subpolysomal fractions (fractions 1-5). Similar results were obtained for cells infected with A5-16, with approximately 34% of the gene 11 mRNA located in the subpolysomal fractions. These data showed that the differences in sedimentation of gene 6 mRNA described above, does not occur globally with all rotavirus mRNA, suggesting that there is differential regulation among the rotavirus genes.

Translational Efficiencies of Specific Rotavirus mRNAs are Differentially Regulated Throughout the Replication Cycle

We next examined the sedimentation of gene 6 and gene 11 mRNAs in the gradients at four hours (figure 4.3) and at six hours (figure 4.4) post infection to determine if differential expression of rotavirus genes was temporally regulated. At four and six hours post infection the results were reversed compared to those observed

at two hours, and the greatest difference between the two strains was in the sedimentation of gene 11 mRNA, not gene 6 mRNA. Polysome analyses were performed as before, except infected cells were harvested at four and six hours post infection. At four hours post infection (Figure 4.3), the majority of NCDV gene 6 mRNA (approximately 65%) was found in the polysomal fractions. This is consistent with the results observed for B641 gene 6 mRNA [297]. Similar results were obtained for cells infected with A5-16, with approximately 65% of gene 6 mRNA located in the polysomal fractions. However, the sedimentation gene 11 mRNA was different between the two strains. Approximately, 83% of NCDV gene 11 mRNA was located in the subpolysomal fractions, suggesting that gene 11 mRNA is very inefficiently translated in NCDV infected cells. In contrast, the sedimentation profile of A5-16 gene 11 mRNA demonstrated more of a bimodal distribution, with RNA detected throughout the gradient. Quantification of the RNA in each fraction showed approximately 59% of the mRNA was located in the subpolysomal fractions. The results observed at six hours post infection were comparable to the results obtained at four hours post infection (figure 4.4). Approximately 57% of gene 6 mRNA was located within the polysomal fractions for both the NCDV and the A5-16 infected cells. A difference in the sedimentation of gene 11 mRNA was observed between infections with the two strains. 73% of NCDV gene 11 mRNA was present in the subpolysomal fractions, compared to 53% of A5-16 gene 11 mRNA. Overall, it appears that NSP5 (encoded by gene 11) is produced more efficiently by the mutant virus throughout the replication cycle, albeit

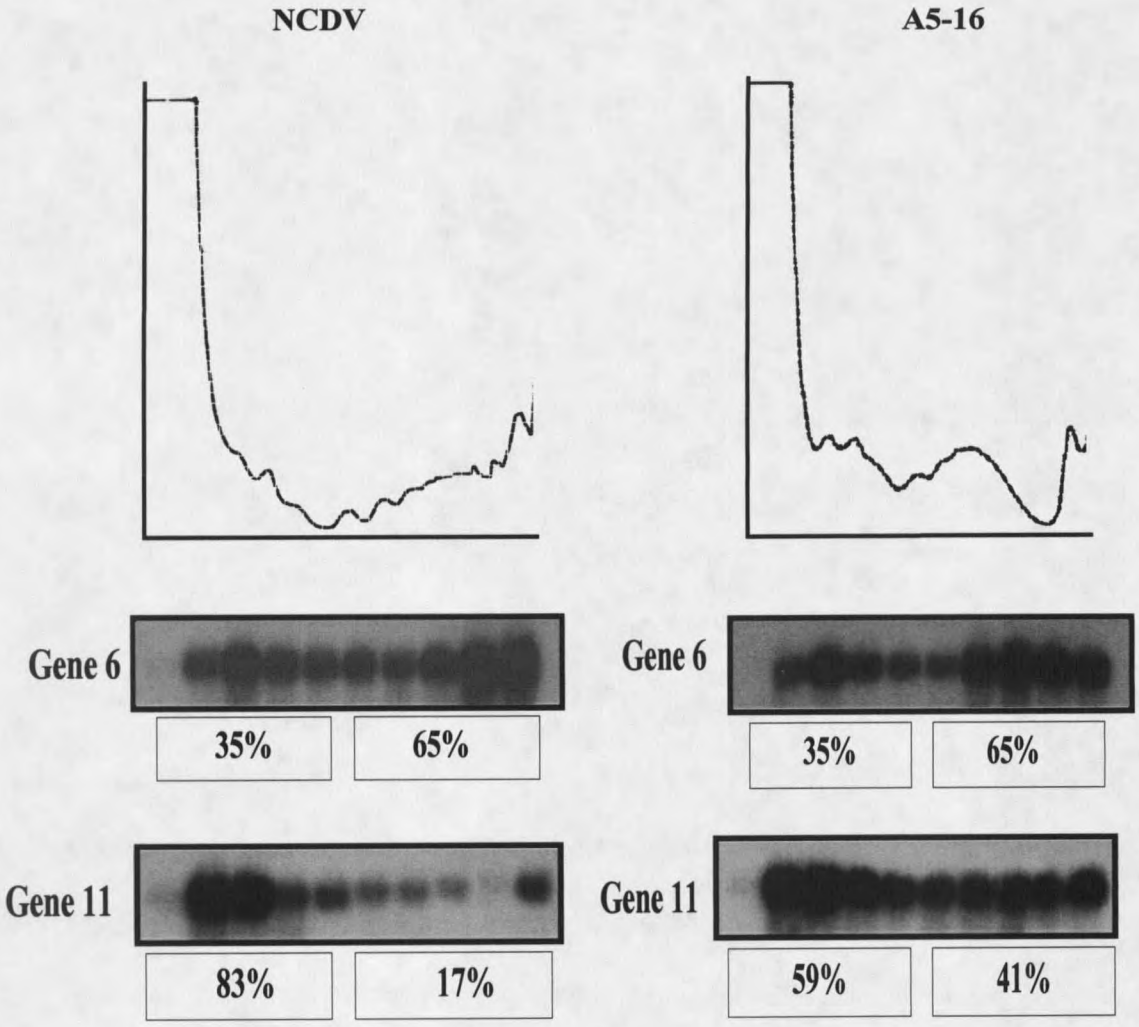



Figure 4.3: Distribution of genes 6 and 11 mRNA in polyribosome gradients 4 hours post infection. NCDV-infected lysates (left panel) or A5-16-infected lysates (right panel) harvested at 4 h p.i. were centrifuged through 0.5-1.5 M linear sucrose gradients for 58 minutes at 159,000 x *g* in a SW55 rotor. The top panels are absorbance profiles. The lower panels show Northern blot hybridization of genes 6 and 11. Radioactive signals were quantified with Bio-Rad Molecular Imager FX with Quantity One software. Percentages of mRNA in polysomal fractions are shown below blots.

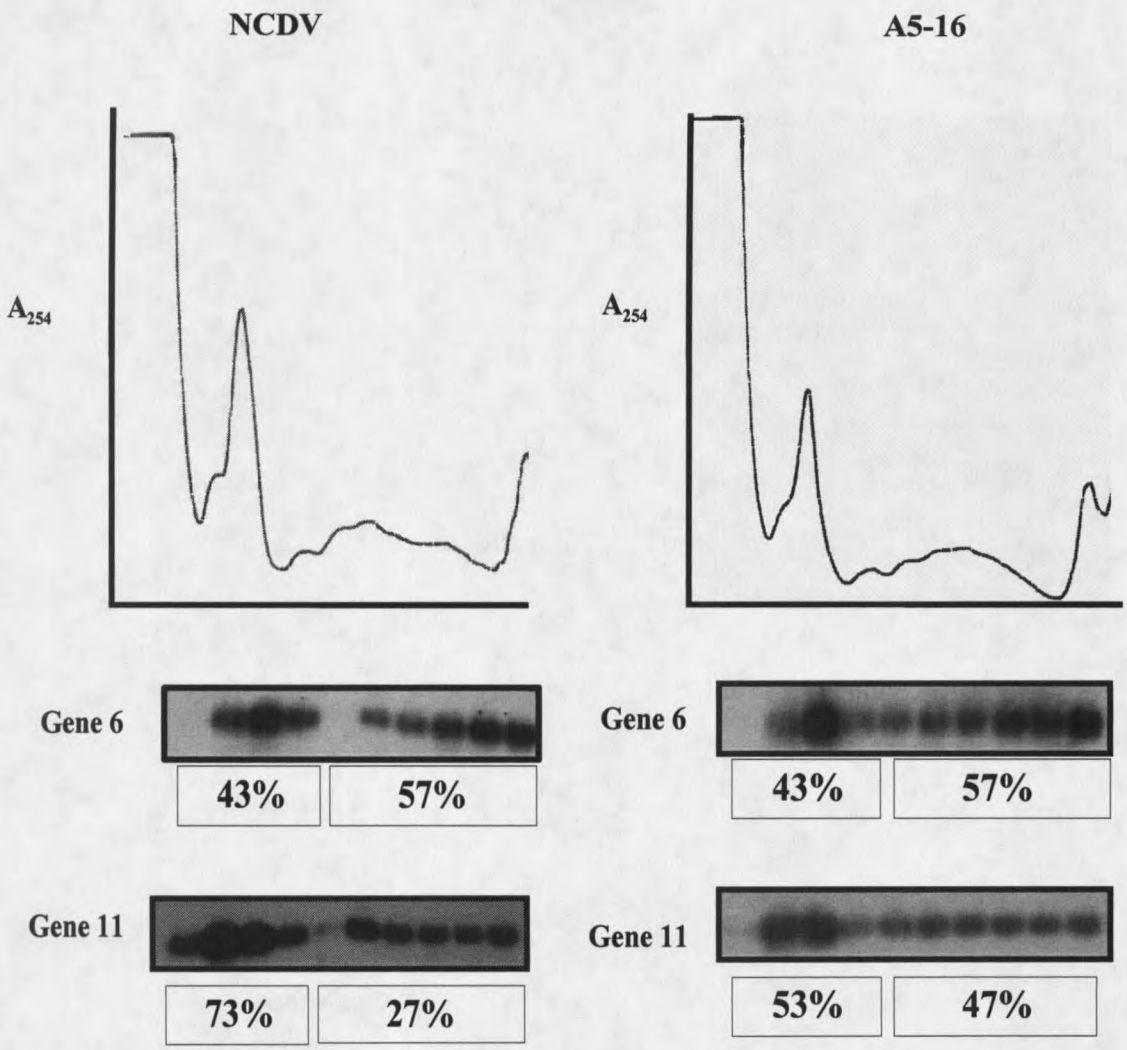



Figure 4.4: Distribution of genes 6 and 11 mRNA in polyribosome gradients 6 hours post infection. NCDV-infected lysates (left panel) or A5-16-infected lysates (right panel) harvested at 6 h p.i. were centrifuged through 0.5-1.5 M linear sucrose gradients for 58 minutes at 159,000 x *g* in a SW55 rotor. The top panels are absorbance profiles. The lower panels show Northern blot hybridization of genes 6 and 11. Radioactive signals were quantified with Bio-Rad Molecular Imager FX with Quantity One software. Percentages of mRNA in polysomal fractions are shown below blots.

less so at the beginning of the replication cycle. Conversely, gene 6 mRNA appears to be translated at the same efficiency at four and six hours post infection, but at two hours post infection the mutant strain synthesizes VP6 more efficiently than NCDV. These results demonstrated that the differential regulation of translational efficiencies of specific viral mRNAs occurs throughout the replication cycle.

Time-Dependent Differences in the Sedimentation of the Viral mRNA Coincides with the Expression of NSP1

NSP3 has been shown to enhance translation of rotavirus mRNA by acting as a PABP analogue [249, 250]. NSP3 is expressed at a higher quantity in A5-16 infected cells than in NCDV infected cells. Therefore, we evaluated the relative amounts of NSP3 at each time point to determine if the differences in the sedimentation of the two genes serving as our models at two, four, and six hours correlated with the differences in the relative amounts of NSP3 between the two strains at these times post-infection (Figure 4.5). Viral proteins were labeled with TRAN$^{35}$S- label for 30 minutes prior to harvesting the cells at the indicated time points. Quantification of NSP3 protein bands showed that NSP3 was present in the A5-16 infected lysates at a two-fold higher amount than NSP3 present in the NCDV infected lysates at two and four hours post-infection. At six hours post-infection, this difference increased to four-fold. The two-fold difference present at two and four hours post infection does not correlate with the differences in the sedimentation of gene 11 mRNA or gene 6 mRNA at these two time points. At two hours post infection, NSP1 was produced at such a low amount that it was not detected on film. At four and six hours post infection, a band representing

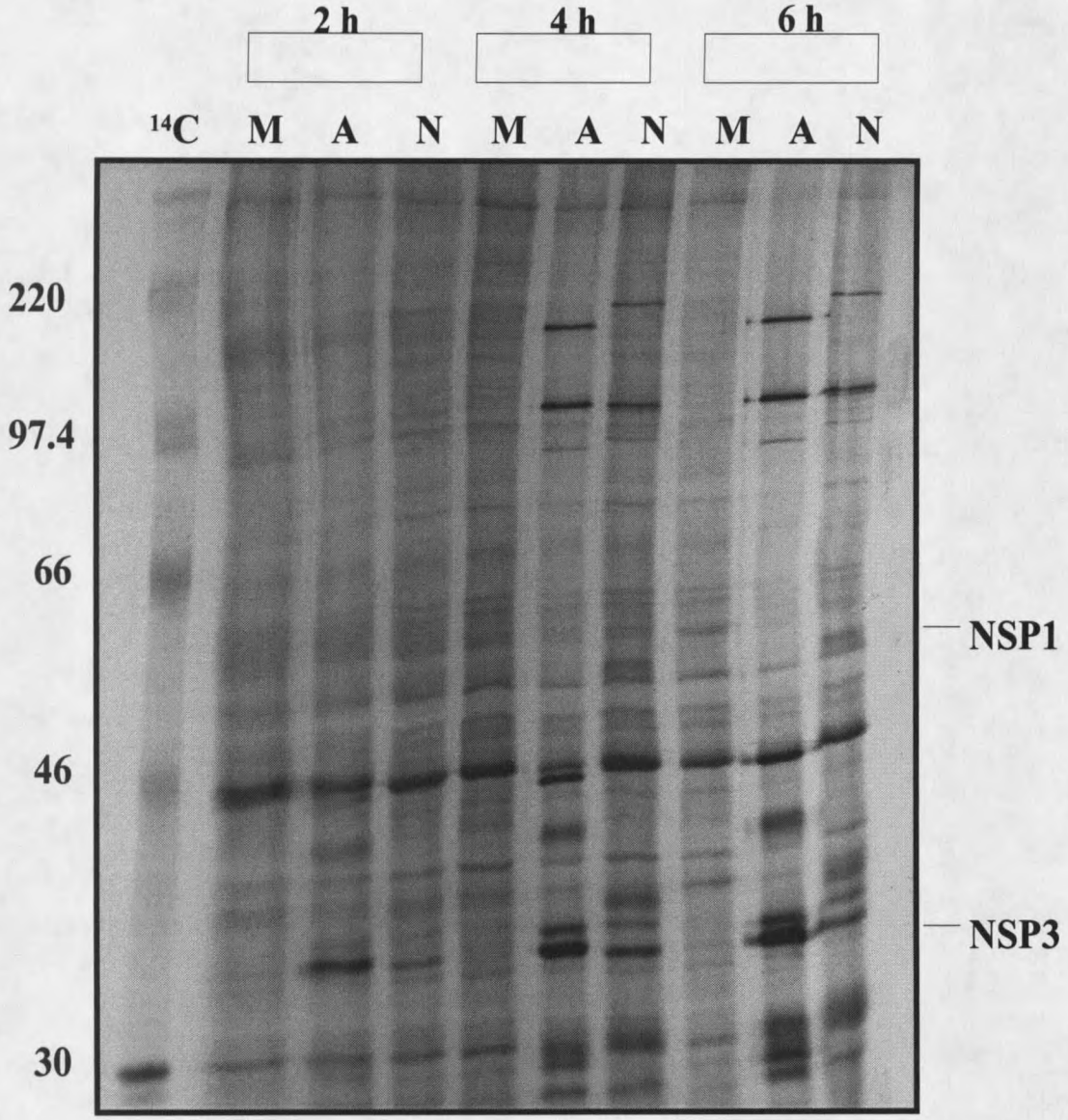



Figure 4.5: Metabolic labelling of rotavirus protein synthesis throughout the replication cycle. MA104 cells in 35 mm dishes were mock-infected (M) or infected with A5-16 (A) or NCDV (N) in the presence of TRAN35S-label and 10μg Actinomycin D ml-1. Cultures were harvested 2 h, 4 h, 6 h p.i. in RIPA bufer. Proteins were seperated by 10% SDS-PAGE and visualized by autoradiography. Rotavirus proteins are indicated. Proteins were quantified with a Bio-Rad Molecular Imager FX with Quantity One software.

NSP1 at approximately 50 kD is detected in the NCDV infected lysates. This time-dependent expression of NSP1 coincides with the differences found in the sedimentation of the genes at two hours when compared to the other time points. These data suggest that NSP3 is not the single regulatory element and that NSP1 maybe important in regulating gene expression at the level of protein synthesis.

Semi-quantitative Analyses Demonstrates Gene 11 mRNA but not Gene 6 mRNA Sediments Differently at Each Time Point

The efficiency of translation is determined by not only the amount of mRNA present in the subpolysomal fraction, but also the amount of mRNA present in the small and large polysomal fractions. Therefore, the sedimentation of the individual viral mRNA in the following three categories: subpolysomal fractions consisting of the 40S and 60S subunit and the 80S ribosome, small polysomes containing 1-4 additional ribosomes, and large polysomes containing 5 or more additional ribosomes was evaluated. Table 4.1 shows the semi-quantitative data of the sedimentation of gene 6 mRNA and gene 11 mRNA for both strains. Gene 6 mRNA was present in the subpolysomal and large polysomal fractions. However, very little to no gene 6 mRNA was present in the small polysomal fractions for either strain. The sedimentation of gene 6 mRNA was the same for both strains at each time point. Therefore, the semi-quantitative analyses of the sedimentation of gene 6 mRNA demonstrated no differences in the translation efficiencies between the strains throughout the replication cycle. Conversely, the sedimentation of gene 11 mRNA differed at each time point. At two hours post-infection, mRNA from A5-16-infected cells showed a moderate amount

present in small polysomes. Gene 11 mRNA was also detected in the subpolysomal and large polysomal fractions. However, a moderate amount of gene 11 mRNA in the wildtype infected cells was present in the subpolysomal fractions and very little to no

Table 4.1. Semi-quantitative analysis of the sedimentation of gene 6 and gene 11 mRNAs for both strains in the polysome gradients. (-) no RNA, (+/-) very little to no RNA, (+,++,+++) represent increasing amounts of RNA.

| | | Gene 6 | | | Gene 11 | | |
|---|---|---|---|---|---|---|---|
| Time (hpi) | Strain | Subpolysomal & mRNP | Small | Large | Subpolysomal & mRNP | Small | Large |
| 2 | Wildtype | + | - | ++ | ++ | + | +/- |
| | Mutant | + | - | ++ | + | + | ++ |
| 4 | Wildtype | + | +/- | ++ | +++ | +/- | - |
| | Mutant | + | +/- | ++ | + | + | + |
| 6 | Wildtype | + | +/- | ++ | +++ | + | +/- |
| | Mutant | + | +/- | ++ | ++ | + | + |

mRNA was present in the large polysomes. At four hours post infection the differences in the sedimentation of gene 11 mRNA became more apparent. Translation of gene 11 in the NCDV infected cells was severely inhibited when compared to the A5-16 infected cells. In the wildtype infection large amounts of gene 11 mRNA was located in the subpolysomal fractions, while little and no mRNA was found in the small and large polysome fractions, respectively, whereas in the A5-16 infected cells, gene 11 mRNA was detected in all three categories. This inhibition of gene 11 expression in the wildtype infection at four hours post-infection coincides with the expression of NSP1 (Figure 4.5). At six hours post infection, the sedimentation profile of gene 11 mRNA

was similar between both strains. In the NCDV infected cells, a large amount of gene 11 mRNA sedimented in the subpolysomal fractions. RNA is also detected in the small polysomes while very little RNA is detected in the large polysomes. A moderate amount of gene 11 mRNA in the A5-16 infected cells was located in the subpolysomal fractions. However, unlike the NCDV infected cells gene 11 mRNA was present in both the small and large polysomal fractions. The similar profile at six hours post-infections could be due to the mRNA being present in subviral particles or replication intermediates. These data further suggest a regulatory role of NSP1. Since the differences occur in gene 11, an mRNA that is inefficiently translated when compared to gene 6 mRNA, and gene 11 is not translated as efficiently in the wildtype infection compared to the mutant infection, these data imply that NSP1 may play a regulatory role in gene expression.

## Discussion

We investigated the possible role of NSP1 in the regulation of viral gene expression at the level of protein synthesis. Using polysome analyses, the data showed a difference in the translation of gene 6 or gene 11 mRNAs between the mutant strain (A5-16), which lacks NSP1, and a wildtype strain (NCDV). At two hours post-infection, the sedimentation of gene 6 mRNA in the polysome gradient differed between the two strains. Gene 11 mRNA varied only slightly between the two strains. However, at four and six hours post infection different results were obtained. The sedimentation of gene 11 mRNA differed between the two strains while the

sedimentation of gene 6 mRNA was similar between the two strains. The differences in the sedimentation of the gene 6 and gene 11 mRNAs between 2 hours post infection and four or six hours post infection coincided with the expression of NSP1. These results suggest a regulatory role of NSP1 in rotavirus gene expression. The semi-quantitative interpretation of the data showed that the distribution of gene 11 mRNA differed between the two strains at each time point. This analysis further demonstrated that at four hours post infection the expression of gene 11 in the wildtype infection was severely inhibited. This inhibition coincides with the expression of NSP1, which insinuates that NSP1 may inhibit viral gene.

Regulation of protein synthesis occurs at the different steps of translation, although most occurs at the initiation step. Studies have shown that sequences contained within the 5' UTR of viral mRNAs play an important role in regulating translation by binding to *trans*-acting factors [243, 300]. NSP1 binds to the 5' UTR of the rotavirus mRNAs in vitro and could therefore function as a *trans*-acting factor in regulating rotavirus protein synthesis by either enhancing or inhibiting the translation of specific viral mRNA. Other RNA-binding proteins that bind to 5' UTRs have been shown to modulate gene expression by either enhancing or inhibiting translation. The influenza virus nonstructural protein, NS1, enhances the translation of viral genes by binding to *cis*-acting sequences in the 5' UTR [243]. This enhancement could be due to NS1 also binding to eIF4G, thereby recruiting the translation initiation machinery [301]. In contrast, other proteins inhibit translation by binding to the 5'UTR. For example, translin binds to the glyceraldehyde 3-phosphate dehydrogenase (Gapds) mRNA just

upstream of the start codon [302]. This translin-mRNA interaction inhibits translation by blocking the translocation of the ribosome or by preventing the recognition of the start codon. Another example is provided by TCP80, a protein that selectively binds mRNA and is thought to inhibit translation by forming untranslatable messenger ribonucleprotein complexes [303].

Although NSP1 binds to all rotavirus 11 mRNAs in vitro, the affinity of NSP1 for each mRNA has not been determined. Therefore, it is possible that NSP1 differentially regulates the expression of the viral mRNA by binding to the individual viral mRNAs with different affinities. Such a mechanism has been shown for influenza virus. The influenza nonstructural protein NS1 binds to the 5' UTR of the M and NP mRNA and to the NS mRNA to a lesser extent [243]. The NS1 protein stimulates translation of the M and NP protein, but not the NS protein [304]. The data obtained from these studies demonstrate that a viral protein could differentially regulate the expression of viral genes by differentially binding to viral mRNA.

Differential rates of translation initiation play an important role in the regulation of protein synthesis. To account for the differences in translation initiation, it was proposed that mRNAs must compete for a limiting message-discriminatory initiation factor in order to be efficiently translated [278]. Studies using reovirus show that the reovirus mRNAs compete with different efficiencies for limiting components of the translational machinery [283, 284]. These differences become apparent when the mRNA-discriminatory component is limited. In order to compete, the mRNAs contain a unique feature that help in determining it's translation initiation efficiency [305]. In

rotavirus, this unique feature could be a *cis*-acting element, perhaps in the 5' UTR that binds to NSP1. The competition among the viral mRNAs for a limiting component could be an important event in determining the translation efficiencies of the individual viral mRNAs [284].

The function of NSP1 is unknown; however, this protein contains characteristics similar to *trans*-acting regulatory proteins. NSP1 contains a zinc finger motif and binds to the 5' UTR of all 11 mRNA in vitro. However, NSP1 is expressed at limiting levels during the replication, and therefore, it is unlikely to bind to all the viral mRNAs that are present. Indirect immunofluorescence assay and subcellular fractionation studies showed that NSP1 is associated with the cytoskeleton. It is known that actively translated mRNAs are bound to the cytoskeleton [306]. These data, along with the data presented in this chapter, suggest that NSP1 may function as a *trans*-acting factor in regulating viral gene expression. Further studies are needed to determine the exact function of NSP1 in regulating viral gene expression.

# CHAPTER FIVE

## SUMMARY AND CONCLUSIONS

Rotavirus is the single most important cause of severe dehydrating diarrhea of mammalian neonates. The genome consists of 11 dsRNA segments that encode 12 proteins (six structural and six nonstructural). Although the structural proteins are well characterized, much remains unknown about the functions of the nonstructural proteins. It is demonstrated that during the replication cycle, the expression of rotavirus genes are under transcriptional and translation control. Today, gaps still exist in understanding the molecular mechanisms and the proteins involved in rotavirus replication, especially in regulation of gene expression. With this in mind, the hypothesis that NSP1 is involved in the regulation of viral gene expression was formulated.

In chapter three, the possible mechanisms involved in the regulation of rotavirus gene expression are investigated. In these studies, metabolic labeling of viral proteins demonstrate that VP6 was expressed at least 10-fold excess over NSP1. Further investigations demonstrate that the difference in the expression of VP6 and NSP1 was not due to a difference in the protein stability, or to a difference in the accumulation of encoding mRNAs. Polysome analyses demonstrated a difference in the translation efficiencies between gene 5 and gene 6 mRNAs. These studies also illustrated that gene 5 mRNA was a poor template for initiating translation when compared to gene 6 mRNA.

In chapter four, the role of NSP1 in regulation of viral gene expression was investigated using a mutant bovine rotavirus strain A5-16, that does not encode NSP1. Polysome analysis showed a difference in the sedimentation of gene 11 mRNA throughout the replication cycle, but the difference was diminished at an early time point post-infection. The reverse effect was observed for gene 6 mRNA, which had the same sedimentation profile for both strains at 4 and 6 hours post infection. However, at two hours post-infection, the sedimentation profile differed between the two strains. The changes apparent in the sedimentation of gene 6 or gene 11 mRNA between 2 hours post infection and the remaining time points coincided with detectable NSP1 levels. These data not only demonstrate that the genes were differentially regulated throughout the replication cycle, but also suggested a role for NSP1 as a regulatory factor in viral gene expression. NSP1 could be a *trans*-acting factor that binds to *cis*-acting sequences contained in the viral mRNA. By interacting with the viral mRNA, NSP1 could enhance or inhibit gene expression. Part of this regulation could be through direct interactions between NSP1 and NSP3 to enhance translation or by interfering with the translocation of the preinitiation complex down the 5' UTR to inhibit translation. The possibility also exists that NSP1 indirectly effects gene expression through interactions that have yet to be described.

In conclusion, the hypothesis that NSP1 is involved in the regulation of viral gene expression was tested. These studies start to elucidate possible mechanisms of translational control during the rotavirus replication cycle. In some instances, the regulation of rotavirus gene expression occurs at the level of translation initiation, and

this regulation could be mediated by NSP1. These are the first studies that demonstrate a possible role of NSP1 in the regulation of translation. Further studies are needed to determine the exact mechanism of how NSP1 regulates viral gene expression. Ultimately, understanding how nonstructural proteins are involved in regulating gene expression and replication will result in a better understanding of the replication cycle, which in turn may lead to new methods of intervention.

# CHAPTER SIX

## FUTURE STUDIES

The studies presented in the previous chapters have demonstrated a mechanism for the regulation of rotavirus gene expression at the level of translation. These studies further suggested a regulatory role for NSP1. However, many questions still exist regarding the mechanisms involved in these processes. How does NSP1 regulate viral gene expression? Does NSP1 enhance or inhibit the expression of a gene? Does NSP1 regulate expression directly or indirectly? What role does the upregulation of NSP3 in the A5-16 strain play in gene expression? In this chapter, studies are proposed to address some of these questions.

The A5-16 strain expresses NSP3 at higher levels than the wildtype strain. This upregulation of the NSP3 expression could be compensating for the lack of the NSP1 expression and thereby skewing the results from the polysome analyses in chapter 4. Therefore, to confirm our results from chapter 4, polysome analysis will be performed using the siRNA system. siRNA has evolved into a powerful tool to investigate gene function [307]. By using the siRNA system, the expression of NSP1 will become knocked out and the virus will not have time to adapt and alter the expression of NSP3. This would eliminate any effect that the upregulation of NSP3 would have on the translation efficiencies of the viral mRNA. Performing polysome analysis and comparing the sedimentation of the mRNA between cells infected with virus expressing NSP1 and not expressing NSP1, will further confirm the results in chapter four.

As suggested by the data in chapter four, NSP1 may play a role, whether direct or indirect, in regulating translation of specific viral genes. However, it is still unknown if NSP1 enhances or inhibits the expression of individual rotavirus genes. To begin to identify the role of NSP1, the affinity of NSP1 for the individual rotavirus mRNAs should be determined. If NSP1 binds to the individual rotavirus genes with different affinities, this data can be compared with the polysome data and a role for NSP1 can be suggested. For example, if NSP1 has a high affinity for a gene, which sediments in the large polysomes (e.g. gene 6), this could suggest that NSP1 acts as an enhancer. However, if NSP1 has a higher affinity for a gene that is inefficiently translated (e.g. gene 5 or gene 11) this could suggest that NSP1 inhibits the expression of that particular gene. *Cis*-acting sequences within the mRNA that NSP1 binds to with the greatest affinity should also be identified. This sequence can be compared among the various rotavirus genes to determine a possible consensus sequence and to predict other genes that NSP1 could regulate. Comparing the affinity of NSP1 for these genes and the sedimentation of these genes in a polysome gradient could further support the regulatory role of NSP1.

A possible mechanism of how NSP1 regulates rotavirus gene expression could be due not only to the interaction with the mRNA, but also to the interaction between NSP1 and NSP3. To determine if the NSP1-NSP3 interaction directly influences the expression of the gene, the binding domains should be determined. NSP1 could hinder gene expression if it binds to the same domains of NSP3 that are required for dimerization, eIF4G1 binding or RNA-binding. However, if NSP1 binds to a different

domain, NSP1 could increase gene expression. This could be determined by performing in vitro translation assays and mutating the NSP1-binding domain of NSP3. If knocking out this interaction changes the expression of the gene, the direct interaction of NSP1 and NSP3 is suggested to control gene expression.

A5-16 overexpresses NSP3 as previously described. It would be interesting to determine why the strain that lacks NSP1 also over expresses NSP3. This over expression could possibly compensate for the lack of NSP1 in the cells. One possible mechanism is by eliminating the competition between the viral genes for NSP3. If the interaction between the mRNA and NSP1 allows for a mRNA to outcompete other mRNAs for NSP3 and the translation machinery, then the lack of NSP1 would disrupt this competition. In doing so, some genes would not be efficiently expressed. Overexpressing NSP3 could eliminate some of the competition by being produced in high enough amounts that the majority of the viral mRNA will bind to NSP3 and become translated. Performing in vitro competition assays would determine if this scenario occurs.

# REFERENCES CITED


1. Flewett TH, Bryden, A.S., Davies, H.A., Woode, G.N., Bridger J.C., And Derrick, J.M.: Relation between viruses from acute gastroenteritis of children and new born calves. Lancet 1974;ii:61-63.

2. Syverton JT, and Olitsky, P.K.: Bacteriological studies on an epizootic of intestinal disease in suckling and newly weaned mice. J Exp Med 1934;60:385-394.

3. Cheever FS, and Mueller, J.H.: Epidemic diarrhoeal disease of suckling mice. I. Manifestation, epidemiology and attempts to transmit the disease. J Exp Med 1947;85:405-416.

4. Pappenheimer AM, and Cheever, F.S.: Epidemic diarrhoeal disease of suckling mice. IV. Cytoplasmic inclusion bodies in intestinal epithelium in relation to the disease. J Exp Med 1948;88:317-24.

5. Pappenheimer AM, and Enders, J.F.: Epidemic diarrhoeal disease of suckling mice. II. Inclusion in intestinal epithelium cells. J Exp Med 1947;85:417-22.

6. Adams WR, and Kraft, L.M.: Electron-microscope study of the intestinal epithelium of mice infected with the agent of epizootic diarrhea of infant mice. Am J Pathol 1967;51:39-44.

7. Adams WR, and Kraft, L.M.: Epizootic diarrhea of infant mice: identification of the etiologic agent. Science 1963;141:359-360.

8. Kraft LM: Studies of the etiology and transmission of epidemic diarrhoea of infant mice. J Exp Med 1957;106:743-755.

9. Mebus CA, Underdahl, N.R., Rhodes, M.B., and Twiehaus, M.J.: Calf diarrhea (scours) reproduced with a virus from a field outbreak. Univ Nebraska Res Bull 1969;223:1-6.

10. Bishop RF, Davidson, G.P., Holmes, I.H., and Ruck, B.J.: Detection of a new virus by electron microscopy of faecal extracts from children with acute gastroenteritis. Lancet 1974;i:149-151.

11. Lecce JG, King, M.W., and Mock, R.: Reovirus-like agent associated with fatal diarrhea in neonatal pigs. Infect Immun 1976;14:816-25.

12. McNulty MS, Pearson, G.R., McFerran, J.B., Collins, D.S., and Allan, G.M.: A reovirus-like agent (rotavirus) associated with diarrhea in neonatal pigs. Vet Microbiol 1976;1:55-63.

13. Flewett TH, Bryden, A.S., and Davies, H.A.: Virus diarrhea in foals and other animals. Vet Rec 1975;96:477.

14. Bryden AS, Thouless, M.E., and Flewett, T.H.: A rabbit rotavirus. Vet Rec 1976;99:323.

15. Reed DE, Daley, C.A., and Shane, H.J.: Reovirus-like agent associated with neonatal diarrhea in pronghorn antelope. J Wild Dis 1976;12:488-91.

16. Snodgrass DR, Smith, W., Gray, E.W., and Herring, J.A.: A rotavirus in lambs with diarrhoea. Res Vet Sci 1976;20:113-114.

17. Tzipori S, Caple, I.W., and Butler, K.: Isolation of rotavirus from deer. Vet Rec 1976;99:398.

18. Woode GN, Bridger, J., Hall, G.A., Jones, J.M., and Jackson, G.: The isolation of reovirus-like agents (rotaviruses) from acute gastroenteritis of piglets. J med microbiol 1976;9:203-209.

19. Yason CV, Summers, B.A., and Schat, K.A.: Pathogenesis of rotavirus infection in various age groups of chickens and turkeys: pathology. Am. J. Vet. Res 1987;48:927-938.

20. DeLeeuw PW, Ellens, D.J., Straver, P.J., VanBalken, J.A.M., Moerman, A. and Baanvinger, T.: Rotavirus infection in calves in dairy herds. Res Vet Sci 1980a;29:135-141.

21. Saif LJ, Redman, D.R., Smith, K.L.,and Theil, K.W.: Passive immunity to bovine rotavirus in newborn calves fed colostrum supplements from immunized or non-immunized cows. Infect Immun 1983;41:1118-31.

22. Acres SD, Saunders, J.R., and Radostist, O.M.: Acute undifferentiated neonatal diarrhea of beef calves: the prevalence of enterotoxigenic *E. Coli*, reo-like (rota) virus and other enteropathogens in cow-calf herds. Can Vet J 1977;18:113-121.

23. Snodgrass RR, Terzolo, H.R., Sherwood, D., Campbell, I., Menzies, J.D., and Synge, B.A.: Aetiology of diarrhoea in young calves. Veterinary Record 1986;119:31-34.

24. Theil KW: Group A Rotaviruses. Boca Raton, CRC Press, 1990.

25. Estes MK: Rotaviruses and their replication, in Knipe DM, Howley, P.M. (ed): Fields Virology. Philadelphia, Lippincott Williams and Wilkins, 2001a, pp 1747-85.

26. Nakata S, Estes, M.K., Graham, D.Y., Loosle, R., Tao, H., Wang, S.H., Saif, L.J., and Melnick, J.L.: Antigenic characterization and ELISA detection of adult diarrhea rotaviruses. J Infect Dis 1986;154:448-455.

27. Yolken R, Wee, S.B., Eiden, J., Kinney, J., and Vonderfecht, S.: Identification of a group reactive epitope of group B rotaviruses recognized by monoclonal antibody and application to the development of a sensitive immunoassay for viral characterization. J Clin Microbiol 1988;26:1853-58.

28. Graham DY, and Estes, M.K.: Proposed working serologic classification system for rotaviruses. Ann Inst Pasteur 1985;136:5-12.

29. Johnson MA, and McCrae, M.A.: Molecular biology of rotaviruses VIII. Quantitative analysis of regulation of gene expression during virus replication. J Virol 1989;63(5):2048-55.

30. Palombo EA: Genetic and antigenic diversity of human rotaviruses: potential impact on the success of candidate vaccines. FEMS Micro Lett 1999;181:1-8.

31. Greenberg HB, McAuliff, V., Valdesuso, J., Wyatt, R., Flores, J., Kalica, A., Hoshino,Y., and Singh, N.: Serological analysis of the subgroup protein of rotavirus using monoclonal antibody. Infect Immun 1982;39:91-99.

32. Taniguchi K, Urasawa, T., Urasawa, S., and Yasuhara, T.: Production of subgroup specific-monoclonal antibodies against human rotaviruses and their application to an enzyme-linked immunosorbent assay for subgroup determination. J Med Virol 1984;14:115-125.

33. Kalica AR, Greenberg, H.B., Espejo, R.T., Flores, J., Sereno, M.M., Kapikian, A.Z., and Chanock, R.M.: Genes of human (strain WA) and bovine (strain UK) rotavirus that code for neutralization and subgroup antigens. Virology 1981;112:385-90.

34. Mota-Hernandez F, Calva, J.J., Gutierrez-Camacho, C., Villa-Contreras, S., Arias, C.F., Padilla-Noriega, Luis., Guiscafre-Gallardo, H., Guerrero, M.D., Lopez, S., Munoz, O., Contreras, J.F., Cedillo, R., Herrera, I., and Puerto, F.I.: Rotavirus diarrhea severity is related to the VP4 type in Mexican children. J Clin Microbiol 2003;41(7):3158-62.

35. Hoshino Y, Sereno, M.M., Midthun, K., Flores, J., Kapikan, A.Z., and Chanock, R.M.: Independent segregation of two antigenic specificities (VP3 and VP7) involved in neutralization of rotavirus infectivity. PNAS 1985;82:8701-4.

36. Offit PA, and Clark, H.F.: Maternal antibody-mediated protection against gastroenteritis in due to rotavirus in newborn mice is dependent on both serotype and titer of antibody. J Infect Dis 1985;152:1152-58.

37. Snodgrass DR, Ojeh, C.K., Campbell, I., and Herring, A.J.: Bovine rotavirus serotypes and their significance for immunization. J Clin Microbiol 1984;20:342-46.

38. Mebus CA, Stair, E.L., Underdahl, N.R., and Twiehaus, M.J.: Pathology of neonatal calf diarrhea induced by reo-like virus. Vet Pathol 1974;8:490-505.

39. Pearson GR, McNulty, M.S., and Logan, E.F.: Pathological changes in the small intestine of neonatal calves naturally infected with reo-like virus (rotavirus). Vet Rec 1978;102:454-58.

40. Torres-Medina A: Effect of combined rotavirus and *Escherichia coli* in neonatal gnotobiotic calves. Am J Vet Res 1984;45(4):643-51.

41. Crouch CF, and Woode, G.N.: Serial studies of virus multiplication and intestinal damage in gnotobiotic piglets infected with rotavirus. J Med Microbiol 1978;11:325-34.

42. Theil KW, Bohl, E.H., Cross, R.F., Kohler, E.M., and Agnes, A.G.: Pathogenesis of porcine rotaviral infection in experimentally inoculated gnotobiotic pigs. Am J Vet Res 1978;39:213-220.

43. Lundgren O, Peregrin, A.T., Persson, K. Kordasti, S. Uhnoo, I., and Svensson L: Role of the enteric nervous system in the fluid and electrolyte secretion of rotavirus diarrhea. Science 2000;287:491-5.

44. Hamilton JR: The pathophysiological basis for viral diarrhea: a progress report. J Ped Gastroent Nutr 1990;11:150-4.

45. Desselberger U: Viral factors determining rotavirus pathogenicity. Arch Virol 1997;13(suppl):131-139.

46. Ball JM, Tian, P., Zeng, C. Q.-Y., Morris, A.P., and Estes, M.K.: Age-dependent diarrhea induced by a rotaviral nonstructural glycoprotein. Science 1996;272:101-104.

47. Estes MK, Kang, G., Zeng, C.Q-Y., Crawford, S.E., and Ciarlet, M.: Pathogenesis of rotavirus gastroenteritis, in Chadwick D, and Goode, J.A. (ed): Gastroenteritis viruses. New York, John Wiley and Sons, LTD., 2001b, vol 238.

48. Zhang M, Zeng, CQ-Y., Morris, A.P., and Estes, M.K.: A functional NSP4 enterotoxin peptide secreted from rotavirus-infected cells. J Virol 2000;74:11663-70.

49. Dong Y, Zeng, C.Q., Ball, J.M., Estes, M.K., and Morris, A.P.: The rotavirus enterotoxin NSP4 mobilizes intracellular calcium in human intestinal cells by stimulating phospholipase C-mediated inositol 1,4,5-triphosphate production. PNAS 1997;94:3960-65.

50. Lorrot M, Martin, S., and Vasseur, M.: Rotavirus infection stimulates the $Cl^-$ reabsortion process across the intestinal brush-border membrane of young rabbits. J Virol 2003;77(17):9305-11.

51. Weclewicz K, Svensson, L., and Kristensson, K.: Targeting the endoplasmic reticulum-associated proteins to axons and dendrites in rotavirus-infected neurons. Brain Res Bull 1998;46:353-60.

52. Lecce JG, King, M.W., and Dorsey, W.E.: Rearing regimen producing piglet diarrhea (rotavirus) and its relevance to acute infantile diarrhea. Science 1978;199:776-8.

53. Saif LJ, and Fernandez,F.M.: Group A rotavirus veterinary vaccines. J Infect Dis 1996;174(suppl 1):s98-106.

54. Acres SD, and Radostits, O.M.: The efficacy of a modified live reo-like virus vaccine and an *E. coli* bacteria for prevention of acute undifferentiated neonatal diarrhea of beef calves. Can Vet J 1976;17:197-212.

55. Bridger JC: A definition of bovine rotavirus virulence. J Gen Virol 1994;75:2807-2812.

56. Saif LJ, and Jackwood,J.: Enteric virus vaccines: theoretical consideration, current status, and future approaches, in Saif LJ, and Fernandez,F.M. (ed): Viral Diarrheas of Man and Animals. Boca Raton, CRC press, 1990, pp 313-29.

57. Saif LJ, and Smith, K.L.: Enteric viral infections of calves and passive immunity. J Dairy Sci 1985;68:206-28.

58. Saif LJ, Smith, K.L., Landmeier, B.J., Bohl, E.H., Theil, K.W., and Todhunter, D.A.: Immune response of pregnant cows to bovine rotavirus immunization. Am J Vet Res 1984;45:49-58.

59. Gianino P, Mastretta, E., Longo, P., Laccisaglia, A., Sartore, M., Russo, R., and Mazzacre, A.: Incidence of noscomial rotavirus infections, symptomatic and asymptomatic, in breast-fed and non-breast-fed infants. J Hospital Inf 2002;50:13-17.

60. Gouet P, Contrepois, M., Dubourguier, H.C., Riou, Y., Scherrer, R., Laporte, J., Vautherot, J.F., Cohen, J., and L'Haridon, R.: The experimental production of diarrhoea in colostrum deprived axenic and gnotoxenic calves with enteropathogenic *escherichia coli*, rotavirus, coronavirus, and in a combined infection of rotavirus and *E. coli*. Ann Rech Vet 1978;9(3):433-40.

61. Moreau MC, Corthier, G., Muller, M.C., Dubos, F., and Raibaud, P.: Relationships between rotavirus diarrhea and intestinal microflora establishment in conventional and gnotobiotic mice. J Clin Microbiol 1986;23(5):863-868.

62. Newsome PM, and Coney, K.A.: Synergistic rotavirus and *Escherichia coli* diarrheal infection of mice. Infect Immun 1985;47(2):573-573.

63. Uhnoo I, Olding-Stenkvist, E., and Kreuger, A.: Clinical features of acute gastroenteritis associated with rotavirus, enteric adenovirus, and bacteria. Archives of Disease in Childhood 1986;61:732-738.

64. Tzipori SR, Makin, T.J., Smith, M.L., And Kraut, F.L.: Clinical manifestation of diarrhea in calves infected with rotavirus and enterotoxigenic *Escherichia coli*. J Clin Microbiol 1981;13:1011-16.

65. Cohen MB: Etiology and mechanisms of acute infectious diarrhea in infants in the United States. J Pediatr 1991;118:S34-S39.

66. Riepenhoff-Talty M, Lee, P.C., Carmody, P.J., Barrett, H.J., and Ogra, P.L.: Age-dependent rotavirus - enterocyte interactions. Proc. Soc. Exp. Biol Med 1982;170:146-154.

67. Wolf JL, Cukor, F., Blacklow, N.R., Dambrauskas, R., and Trier, J.A.: Susceptibility of mice to rotavirus infection:effects of age and administration of corticosteroids. Infect Immun 1981;33:565-74.

68. Moon HW, Kemeny, G., Lambert, G., Stark, S.L. and Booth, G.D.: Age-dependent resistance to transmissible gastroenteritis of Swine. III. Effects of epithelial cell kinetics on coronavirus production and on atrophy of intestinal villi. Vet Pathol 1975;12:434-445.

69. Moon HW: Epithelial cell migration in the alimentary mucosa of the suckling pig. Proc. Soc. Exp. Biol Med 1971;137:151-154.

70. Friedman MG, Galil, A., Sarov, B., Margalith, M., Katzir, G., Midthun, K., Taniguchi, K., Urasawa, S., Kapikan, A.Z., Edelman, R., And Sarov, I.: Two sequential outbreaks of rotavirus gastroenteritis: evidence for symptomatic and asymptomatic reinfections. J Infect Dis 1988;158:814-22.

71. Chiba S, Nakata, S., Urasawa, T., Urasawa, S., Yokoyama, T., Morita, Y., Taniguchi, K., and Nakao, T.: Protective effect of naturally acquired homotypic and heterotypic rotavirus antibodies. Lancet 1986;i:417-21.

72. Kapikian AZ, Kim, H.W., Wyatt, R.G., Cline,L., Arrobio, J.O., Brandt, C.D., Rodriguez, W.J., Sack, D.A., Chanock, R.M., and Parrott, R.H.: Human reovirus-like agent as the major pathogen associated with "winter" gastroenteritis in hospitalized infants and young children. N Engl J Med 1976;294(18):965-72.

73. Glass RI, Kilgore, P.E., Holman, R.C., Jin, S., Smith, J.A., Woods, P.A., Clarke, M.J., Ho, M.S., and Gentsch, J.R.: The epidemiology of rotavirus diarrhea in the United States: surveillance and estimates of disease burden. J Infect Dis 1996a;174(suppl):S5-11.

74. Glass RI, Lew, J.F., Gangarosa, R.E., LeBaron, C.W., and Ho, M.S.: Estimates of morbidity and mortality rates for diarrheal diseases in American children. J Pediatr 1991;118:S27-33.

75. Glass RI, Lang, D.R., Ivanoff, B.N., and Compans, R.W.: Introduction: rotavirus - from basic research to a vaccine. J Infect Dis 1996b;174:S1-2.

76. Ciarlet M, and Estes, M.K.: Interactions between rotavirus and gastrointestinal cells. Curr Opin Microbiol 2001;4:435-441.

77. Parashar UD, Hummelman E.G., Bresee, J.S., Miller, M.A., and Glass, R.I.: Global illness and deaths caused by rotavirus disease in children. Emerg Infect Dis 2003;9(5):565-72.

78. Harrison MS: Rotavirus: An overview - from discovery to vaccine. Ped Nurs 1998;24(4):317-22.

79. Kapikan A: A rotavirus vaccine for prevention of severe diarrhoea of infants and young children: development, utilization and withdrawal. Novartis Found Symp 2001;238:153-79.

80. Pediatrics AAo: Prevention of rotavirus disease: Guidelines for use of rotavirus vaccine. Pediatrics 1998;102(6):1483-91.

81. Kombo LA, Gerber, M.A., Pickering, L.K., Atreya, C.D., Breiman, R.F.: Intussusception, infection, and immunization: summary of a workshop on rotavirus. Pediatrics 2001;108(2).

82. Jin Q, Ward, R.L., Knowlton, D.R., Gabbay, Y.B., Linhares, A.C., Rappaport, R., Woods, P.A., Glass, R.I., and Gentsch, J.R.: Divergence of VP7 genes of G1 rotaviruses isolated from infants vaccinated with reassortment rhesus rotaviruses. Arch Virol 1996;141:2057-76.

83. Kapikian AZ, Hoshino, Y., Chanok, R.M., and Perez-Schael, I.: Efficacy of a quadrivalent rhesus rotavirus-based human rotavirus vaccine aimed at preventing severe rotavirus diarrhea in infants and young children. J Infect Dis 1996;174(suppl):S56-72.

84. Epsul C, Cuello, H., Martenez, N., Centorbi, O., O'Ryan, M., Jackson, L. Campos, F., and Matson, D.O.: Genomic and antigenic variation among rotavirus strains circulating in a large city of Argentina. J Med Virol 2000;61:504-9.

85. McNulty MS: Rotaviruses. Journal of General Virology 1978;40:1-18.

86. Bridger JC, and Woode, G.N.: Neonatal calf diarrhoea: identification of reovirus-like (rotavirus) agent in feces by immunofluorescence and immune electron microscopy. Br Vet J 1975;131:528-35.

87. Mebus CA, White, R.G., Baas, E.P., and Twiehaus, M.J.: Immunity to neonatal calf diarrhea virus. J Am Vet Med Assoc 1973;163:880-3.

88. Schlafer DH, and Scott, F.W.: Prevalence of neutralizing antibody to the calf rotavirus in New York. Cornell Vet 1979;69:262-71.

89. Myers LL, and Snodgrass, D.R.,: Colostral and milk antibody titers in cows vaccinated with a modified live rotavirus-coronavirus vaccine. JAVMA 1982;181:486-88.

90. Schusser G, Hinaidy, B., and Burki, F.: A follow-up study on bovine rotavirus dissemination among calves of a large dairy herd. Microbiologica 1982;5:321-332.

91. McNulty MS, and Logan, E.F.: Longitudinal survey of rotavirus infection in calves. Vet Rec 1983;113:333-335.

92. Thurber ET, Bass, E.P., and Beckenhauer, W.T.: Field trial evaluation of a reo-coronavirus calf diarrhea vaccine. Can J Comp Med 1977;41:131-36.

93. DeLeeuw PW, Ellens, D.J., Talmon, F.P., Zimmer, G.N., and Kommerji, R.: Rotavirus infections in calves: efficacy of oral vaccination in endemically infected herds. Res Vet Sci 1980b;29:142-47.

94. Snodgrass DR, Fahey, K.J., Wells, P.W., Campbell, I., and Whitelaw, A.: Passive immunity in calf rotavirus infections: maternal vaccination increases and prolong immunoglobulin G1 antibody secretion in milk. Infect Immunol 1980;28:344-49.

95. Brussow H, Walther, I., Fryder, V., Sidoti, J., and Bruttin, A.: Cross-neutralizing antibodies induced by single serotype vaccination of cows with rotavirus. J Genet Virol 1988;69:1647-58.

96. Rousic S, Klein, N., Houghton, S., and Charleston, B.: Use of colostrum from rotavirus-immunized cows as a single feed to prevent rotavirus-induced diarrhea in calves. Vet Rec 2000;147:160-61.

97. McNulty MS, and Logan, E.F.: Effect of Vaccination of the dam on rotavirus infection in young calves. Vet Rec 1987;120:250-52.

98. Bellinzoni RC, Blackall, J., and Baro, N.: Efficacy of an inactivated oil-adjuvanted rotavirus vaccine in the control of calf diarrhea in beef herds in Argentina. Vaccine 1989;7:263-69.

99. Cornaglia EM, Fernandez, F.M., Gottschalk, M., Barrandeguy, M.E., Luchel, A., Pasini, M.I., Saif, L.J., Parraud, J.R., Romat, A., and Schudel, A.A.: Reduction in morbidity due to diarrhea in nursing beef calves by use of an inactivated oil-adjuvanted rotavirus *Escherichia coli* vaccine in the dam. Vet Microbiol 1992;30:191-202.

100. Besser TE, Gay, C.C., McGuire, T.C., and Everman, J.F.: Passive immunity to bovine rotavirus infection associated with transfer of serum antibody into the intestinal lumen. J Virol 1988;62:2238-42.

101. Waltner-Towes D, Martin, S.W., Meek, A.H., McMillan, I., and Crouch, G.F.: A field trial to evaluate the efficacy of a combined rotavirus-coronavirus *Escherichia coli* vaccine in dairy cattle. Can J Comp 1988;49:1-9.

102. Martin ML, Palmer, E.L., and Middleton, P.J.: Ultrastucture of infantile gastroenteritis virus. Virology 1975;68:146-53.

103. Roseto A, Escaig, J., Delain, G., Cohen, J., and Scherrer, R.: Structure of rotaviruses as studies by the freeze-drying technique. Virology 1979;98:471-75.

104. Prasad BV, Burns, J.W., Marietta, E., Estes, M.K., and Chiu, W.: Localization of VP4 neutralization sites in rotavirus by three-dimensional cryo-electron microscopy. Nature 1990;343:476-79.

105. Prasad BV, Wang, G.J., Clerx, J.P., and Chiu, W.: Three-dimensional structure of rotaviruses. J Mol Biol 1988;199:269-75.

106. Estes MK: Rotaviruses and their replication, in Fields BN, Knipe, D.M., Howley, P.M. (ed): Fundamental Virology, 3rd Edition. Philadelphia, Lippincott-Raven, 1996, pp 731-61.

107. Shaw AL, Rothnagel, R., Chen, D., Ramig, R.F., Chiu, W., and Prasad, B.V.V.: Three-dimensional visualization of the rotavirus hemagglutinin structure. Cell 1993;74:693-701.

108. Yeager M, Berriman, J.A., Baker, T.S., and Bellamy, A.R.: Three-dimensional structure of the rotavirus hemagglutinin VP4 by cryo-electron microscopy and difference map analysis. EMBO J 1994;13(5):1011-18.

109. Zeng CQ-Y, Wentz, M.J., Cohen, J., Estes, M.K., and Ramig, R.F.: Characterization and replicase activity of double-layered and single-layered rotavirus-like particles expressed from baculovirus recombinants. J Virol 1996;70:2736-42.

110. Prasad BV, Rothnagel, R., Zeng, C.Q., Burns, J.W., Marietta, E., Estes, M.K. and Chiu, W.: Visualization of ordered genomic RNA and localization of transcriptional complexes in rotavirus. Nature 1996;382:471-73.

111. Lawton JA, Estes, M.K., and Prasad, B.V.: Comparative structural analysis of transcriptionally competent and incompetent rotavirus-antibody complexes. PNAS 1999;96:5428-5433.

112. Lawton JA, Zeng, C.Q., Mukherjee, S.K., Cohen, J., Estes, M.K., and Prasad, B.V.: Three-dimensional structural analysis of recombinant rotavirus-like particles with intact and amino-terminal-deleted VP2: implications for the architecture of the VP2 capsid layer. J Virol 1997a;71:7353-60.

113. Newman JFE, Brown, F., Bridger, J.C., and Woode, G.N.: Characterization of a rotavirus. Nature 1975;258:631-33.

114. Chan WK, Au, K.S., and Estes, M.K.: Topography of the simian rotavirus nonstructural glycoprotein (NS28) in the endoplasmic reticulum membrane. Virology 1988;164:435-442.

115. Mattion NM, Cohen, J., and Estes, M.K.: Expression of rotavirus proteins encoded by alternative open reading frames of genome segment 11. Virology 1991;181:295-304.

116. Rixon F, Taylor, P., and Desselberger, U.: Rotavirus RNA segments sized by electron microscopy. J Gen Virol 1984;65:233-39.

117. Estes MK, and Cohen, J.: Rotavirus gene structure and function. Microbiol Rev 1989;53(4):410-49.

118. Imia M, Akatani, K., Ikegami, N., and Furuichi Y.: Capped and conserved terminal structures in human rotavirus genome double stranded RNA segments. J Virol 1983;47:125-136.

119. McCrae MA, and McCorguodale, J.G.: Molecular biology of rotavirus V. Terminal structure of viral RNA species. Virology 1983;126:204-12.

120. Desselberger U, and McCrae, M.A.: The rotavirus genome, in Ramig RF (ed): Rotaviruses. Current topics in microbiology and immunology. London, Springer-Verlag, 1994, vol 185, pp 31-66.

121. Krishna Mohan KV, and Atreya, C.D.: Nucleotide sequence analysis of rotavirus gene 11 from two tissue cultured-adapted ATCC strains, RRV and Wa. Virus Genes 2001;23(2):321-29.

122. Cohen J, Charpilienne, A., Chilmonczyk, S., and Estes, M.K.: Nucleotide sequence of bovine rotavirus gene 1 and expression of the gene in baculovirus. Virology 1989;171:130-40.

123. Estes MK, Graham, D.Y., and Mason, B.B.: Proteolytic enhancement of rotavirus infectivity: molecular mechanism. J Virol 1981;39(3):879-88.

124. Kalica AR, Flores, J., and Greenberg, H.B.: Identification of the rotaviral gene that codes for hemagglutination and protease-enhanced plaque formation. Virology 1983;125:194-205.

125. Espejo RT, Lopez, S., and Arias, C.F.: Structural polypeptides of simian rotavirus SA11 and the effect of trypsin. J Virol 1981;37:156-60.

126. Crawford SE, Mukherjee, S.K., Estes, M.K., Lawton, J.A., Shaw, A.L., Ramig, R.F., and Prasad, B.V.: Trypsin cleavage stabilizes the rotavirus VP4 spike. J Virol 2001;75:6052-61.

127. Ludert JE, Feng, N., Yu, J.H., Broome, R.L., Hoshino, Y., and Greenberg, H.B.: Genetic mapping indicates that VP4 is the rotavirus cell attachment protein in vitro and in vivo. J Virol 1996;70:487-93.

128. Graham KL, Halasz, P., Tan, Y., Hewish, M., Takada, Y., Mackow, E., Robinson, M., and Coulson B.: Integrin-Using Rotaviruses Bind alpha2Beta1 Integrin and alpha2 I Domain via VP4 DGE Sequence and Recognize alphaXBeta2 and alphaVBeta3 by using VP7 during Cell Entry. J Virol 2003;77(18):9969-9978.

129. Dormitzer PR, Sun, Z.J., Blixt, O., Paulson, J.C., Wagner, G., and Harrison, S.C.: Specificity and affinity of sialic acid binding by the rhesus rotavirus VP8* core. J Virol 2002;76(20):10512-17.

130. Hewish MJ, Takada, Y., and Coulson, B.S.: Integrins $\alpha 2\beta 1$ and $\alpha 4\beta 1$ can mediate SA11 rotavirus attachment and entry into cells. J Virol 2000;74:228-236.

131. Dowling W, Denisova, E., LaMonica,R., and Mackow, E.R.: Selective membrane permeabilization by rotavirus VP5* protein is abrogated by mutation in an internal hydrophobic domain. J Virol 2000;74(14):6368-76.

132. Denisova.E.W. D, R., LaMonica R., Shaw, R., Scarlata, S., Ruggeri, F., and Mackow,E.R.: Rotavirus capsid protein VP5* permeabilizes membranes. Journal of Virology 1999;73(3147-53).

133. Keljo DJ, and Smith, A.K.: Characterization of binding of simian rotavirus SA-11 to cultured epithelial cells. J Ped Gastroent Nutr 1988;7:249-56.

134. Petrie BL, Graham, D.Y., and Estes, M.K.: Identification of rotavirus particle types. Intervirology 1981;16:20-28.

135. Suzuki H, Kitaoka,S., Konno, Y., Sato, T., and Ishida, N.: Two modes of human rotavirus entry into MA104 cells. Arch Virol 1985;85:25-34.

136. Suzuki H, Kitaoka,S., Sato, T. Konno, Y., Iwasaki, Y., Numazaki, N., and Ishida, N.: Further investigation on the mode of entry of human rotavirus into cells. Arch Virol 1986;91(134-44).

137. Kohli E, Pothier, P., Tosser, G., Cohen, J., Sandino, A.M., and Spenser, E.: In vitro reconstitution of rotavirus transcriptional activity using viral cores and recombinant baculovirus expressed VP6. Arch Virol 1993;133:451-458.

138. Poch O, Sauvaget, I., Delarue, M., and Tordo, N.: Identification of four conserved motifs among the RNA-dependent polymerase encoding elements. EMBO 1989;8:3867-74.

139. Liu M, Mattion, N.M., and Estes, M.K.: Rotavirus VP3 in insect cells possesses guanyltransferase activity. Virology 1992;188:77-84.

140. Pizarro JL, Sandino, A.M., Pizarro, J.M., Fernandez, J., and Spencer, E.: Characterization of rotavirus guanyltransferase activity associated with poypeptide VP3. J Gen Virol 1991;72:325-332.

141. Prasad BV, Crawford, S., Lawton, J.A., Pesavento, J., Hardy, M., and Estes, M.K.: Structural studies on gastroenteritis viruses. Novartis Found Symp 2001;238:26-46.

142. Lawton JA, Estes, M.K., and Prasad, B.V.: Mechanisms of genome transcription in segmented dsRNA viruses. Adv Vir Res 2000;55:185-229.

143. Sandino AM, Jashes, M., Faundez, G., and Spencer, E.: Role of the inner protein capsid on in vitro human rotavirus transcription. J Virol 1986;60:797-802.

144. Richardson SC, Mercer, L.E., Sonza, S., and Holmes, I.H.: Intracellular localization of rotaviral proteins. Arch Virol 1986;88:251-64.

145. Patton JT: Structure and function of the rotavirus RNA-binding proteins. J Gen Virol 1995;76:2633-44.

146. Gallegos CO, and Patton, J.T.: Characterization of rotavirus replication intermediates: a model for the assembly of single-shelled particles. Virology 1989;172:616-27.

147. Patton JT, and Gallegos, C.O.: Rotavirus RNA replication: single-strand RNA extends from the replicase particle. J Gen Virol 1990;71:1087-94.

148. Chen D, Zeng, C.Q., Wents, M.J., Gorziglia,M., Estes, M.K., and Ramig, R.F.: Template-dependent in vitro replication of rotavirus RNA. J Virol 1994;68:7030-39.

149. Acs G, Schonberg, H., Christman, J., Levin, D.H., and Silverstein, S.C.: Mechanism of reovirus double-stranded ribonucleic acid synthesis in vivo and in vitro. J Virol 1971;8:864-69.

150. Patton JT, and Gallegos, C.O.: Structure and protein composition of the rotavirus replicase particle. Virology 1988;166:358-65.

151. Taraporewala ZF, and Patton, J.T.: Identification and characterization of the helix destabilizing activity of rotavirus nonstructural protein NSP2. J Virol 2001;75:4519-4527.

152. Berois M, Sapin, C., Erk, I., Poncet, D., and Cohen, J.: Rotavirus nonstructural protein NSP5 interacts with major core protein VP2. J Virol 2003;77:1757-63.

153. Afrikanova I, Fabbretti, E., Miozzo, M.C., and Burrone, O.R.: Rotavirus NSP5 phosphorylation is up-regulated by interaction with NSP2. J Gen Virol 1998;79:2679-86.

154. Altenburg BC, Graham, D.Y., and Estes, M.K.: Ultrastructural studies of rotavirus replication in cultured cells. J Gen Virol 1980;46:75-85.

155. Patton JT, Wentz, M., Xiaobo, J., and Ramig, R.F.: cis-Acting signals that promote genome replication in rotavirus mRNAs. J Virol 1996b;70:3961-71.

156. Wentz MJ, Patton, J.T., and Ramig, R.F.: The 3'-terminal consensus sequence of rotavirus mRNA is the minimal promoter for negative-strand RNA synthesis. J Virol 1996;70:7833-41.

157. Patton JT, and Spencer, E.: Genome replication and packaging of segmented double-stranded RNA viruses. Virology 2000;277(2):217-25.

158. Dever TE: Gene-specific regulation by general translation factors. Cell 2002;108:545-556.

159. Gross M, Rubino, M.S., and Hessefort, S.M.: The conversion of eIF2-GDP to eIF2-GTP by eIF2B requires Met-tRNA (fMet). Biochem Biophys Res Commun 1991;181:1500-07.

160. Voorma HO, Adri, A.M., Van Heughten, T., and Van Heughten, H: Initiation of protein synthesis in eukaryotes. Mol Biol Rep 1994;19:139-145.

161. Sonenberg N, Morgan, M.A., Merrick, W.C., and Shatkin, A.J.: A polypeptide in eukaryotic initiation factors that crosslinks specifically to the 5'-terminal cap in mRNA. PNAS 1978(75):4843-47.

162. Duncan R, Milburn, S.C., and Hershey, J.W.B.: Regulated phosphorylation and low abundance of HeLa cell initiation factor eIF-4F suggests a role in translational control. J Biol Chem 1987;262:380-88.

163. Rozen F, Edery, I., Meerovitch, K., Dever, T.E., Merrick, W.C., and Sonenberg, N.: Bidirectional RNA helicase activity of eucaryotic translation initiation factors 4A and 4F. Mol Cell Biol 1990;13:7358-63.

164. Hershey JWB, and Merrick, W.C.: The pathway and mechanism of initiation of protein synthesis, in Sonenberg N, Hershey, J. and Mathews M.B. (ed): Translational Control of Gene Expression. Cold Spring Harbor, Cold Spring Harbor Laboratory Press, 2000, pp 33-88.

165. Sachs AB, Sarnow, P., and Hentze, M.W.: Starting at the beginning, middle, and end: Translation initiation in eukaryotes. Cell 1997;89:831-38.

166. Kozak M: The scanning model for translation: an update. J Cell Biol 1989;108:229-241.

167. Kozak M: Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell 1986;44:283-292.

168. Kozak M: How do eucaryotic ribosomes select initiation regions in messenger RNA? Cell 1978;15:1109-23.

169. Chakrabarti A, and Maitra, U.: Function of eukaryotic initiation factor 5 in the formation of an 80 S ribosomal polypeptide chain initiation complex. J Biol Chem 1991;266:14039-45.

170. Browne GJ, and Proud, C.G.: Regulation of peptide-chain elongation in mammalian cells. Eur. J. Biochem 2002;269:5360-68.

171. Garrett RH, and Grisham, C.M.: Protein synthesis and degradation, in Gruliow B, and Fitz-Hugh, S. (ed): Biochemistry. New York, Saunders College Publishing Harcourt Brace College Publishers, 1995, pp 1040-74.

172. Pape T, Wintermeyer, W., and Rodnina, M.V.: Complete kinetic mechanism of elongation factor Tu -dependent binding of aminoacyl-tRNA to the A site of the *E.coli* ribosome. EMBO 1998;17:7490-97.

173. Ramakrishnan V: Ribosome structure and the mechanism of translation. Cell 2002;108:557-72.

174. Kinzy TG, Ripmaster, T.L., and Woolford, J.L. Jr.: Multiple genes encode the translation elongation factor 1-γ in Saccharomyces cerevisiae. Nucleic acids res 1994;22:2703-07.

175. Moazed D, and Noller, H.F.: Intermediate states in the movement of transfer RNA in the ribosome. Nature 1989;342:142-48.

176. Rodnina MV, Savelsbergh, A., Katunin, V.I., and Wintermeyer, W.: Hydrolysis of GTP by elongation factor G drives rRNA movement on the ribosome. Nature 1997;385:37-41.

177. Nakamura Y, and Ito, K.: Making sense of mimic in translation termination. Trends Biochem Sci 2003;28:99-105.

178. Song H, Mugnier, P., Das, A.K., Webb, H.M., Evans, D.R., Tuite, M.F., and Hemmings, B.A.: The crystal structure of human eukaryotic release factor eRF1 - mechanisms of stop codon recognition and peptidyl-tRNA hydrolysis. Cell 2000;100:311-21.

179. Zhouravleva G, Frolova, L., LeGoff, X., LeGuellec, R., Inge-Vechtomov, S., Kisselev, L., and Phillippe, M.: Termination of translation in eukaryotes is governed by two interacting polypeptide chain release factors, eRF1 and eRF3. EMBO 1995;14:4065-72.

180. Kisselev L, Ehrenberg, M., and Frolova, L.: Termination of translation: interplay of mRNA, rRNAs, and release factors? EMBO 2003;22:175-82.

181. Freistroffer DV, Pavlov, M.Y., MacDougall, J., Buckingham, R.H., and Ehrenberg, M.: Release factor RF3 in *E. coli* accelerates the dissociation of release factors RF1 and RF2 from the ribosome in a GTP-dependent manner. EMBO 1997;16:4126-33.

182. Kisselev L, and Buckingham, R.H.: Translation termination comes of age. Trends Biochem Sci 2000;25:561-67.

183. Jacob FC, and Monod, J.: Genetic regulatory mechanisms in the synthesis of proteins. J Mol Biol 1961;3:318-56.

184. Mathews MB, Sonenberg, N., and Hershey, J.W.B: Origins and principles of translational control, in Sonenberg N, Hershey, J. and Mathews M.B. (ed): Translational control of gene expression. Cold Spring Harbor, Cold Spring Harbor Laboratory Press, 2000, pp 1-31.

185. Arrick BA, Lee, A.L., Grendell, R.L., and Dernyck, R.: Inhibition of translation of transforming growth factor- β3 mRNA by its 5' untranslated region. Mol Cell Biol 1991;11:4306-13.

186. Falcone D, and Andrews, D.W.: Both the 5' untranslated region and the sequences surrounding the start site contribute to efficient initiation of translation in vitro. Mol Cell Biol 1991;11:2656-64.

187. Kozak M: Structural features in eukaryotic mRNAs that modulate the initiation of translation. J. Biol. Chem 1991;226:19867-19870.

188. Dabrowski C, and Alwine, J.C.: Translational control of synthesis of simian virus 40 late proteins from polycistronic 19S late mRNA. J Virol 1988;62:3182-92.

189. Lawson TG, Cladaras, M.H., Ray, B.K., Lee, K.A., Abramson, R.D., and Merrick W.C.: Discriminatory interaction of purified eukaryotic initiation factors 4F plus 4A with the 5' ends of reovirus messenger RNAs. J Biol Chem 1988;263:7266-76.

190. Kozak M: Downstream secondary structure facilitates recognition of initiator codons by eukaryotic ribosomes. PNAS 1990;87:8301-05.

191. Kaspar RL, Kakegawa, T., Cranston, H., Morris, D.R., and White, M.W.: A regulatory cis element and a specific binding factor involved in the mitogenic control of murine ribosomal protein L32 translation. J Biol Chem 1992;267:508-14.

192. Belsham GJ, and Sonenberg, N.: RNA-protein interactions in regulation of picornavirus RNA translation. Microbiol Rev 1996;60:499-511.

193. Lawson TG, Ray, B.K., Dodds, J.T., Grifo, J.A., Abramson, R.D., Merrick W.C., and Thach, R.E.: Influence of 5' proximal secondary structure on the translational efficiency of eukaryotic mRNAs and on their interaction with initiation factors. J Biol Chem 1986;261:13979-89.

194. Sonenberg N: mRNA translation: influence of the 5' and 3' untranslated regions. Curr Opin Genet Dev 1994;4:310-15.

195. Chung S, Eckrich, M., Perrone, B.N., Kohn, D.T., and Furneaux, H.: The Ela-like proteins bind to a conserved regulatory element in the 3'-untranslated region of GAP-43 mRNA. J Biol Chem 1997;272:6593-98.

196. Craig AW, Haghighat, A., Yu, A.T., and Sonenberg, N.: Interaction of polyadenylated-binding protein with the eIF4G homologue PAIP enhances translation. Nature 1998;392:520-23.

197. Kozak M: Possible role of flanking nucleotides in recognition of the AUG initiator codon by eukaryotic ribosomes. Nucleic Acids Res 1981;9:5233-52.

198. Kozak M: Translation of insulin-related polypeptides from messenger RNAs with tandemly reiterated copies of the ribosome binding site. Cell 1983;34:971-78.

199. Banerjee AK: 5'-Terminal cap structure in Eucaryotic messenger ribonucleic Acids. Microbiol Rev 1980;44:175-205.

200. Jackson RJ: Cytoplasmic regulation of mRNA function: the importance of the 3' untranslated region. Cell 1993;74:9-14.

201. Decker CJ, and Parker, R.: A turnover pathway for both stable and unstable mRNAs in yeast: Evidence for a requirement for deadenylation. Genes Dev 1993;7:1632-43.

202. Jackson RJ, and Standart, N.: Do the poly(A) tail and 3' untranslated region control mRNA translation. Cell 1990;62:15-24.

203. Tarun SZ, Jr., and Sachs, A.B.: A common function for mRNA 5' and 3' ends in translation initiation in yeast. Genes Dev 1995;9:2997-3007.

204. Preiss T, and Hentze, M.W.: Dual function of the messenger RNA cap structure in poly(A)-tail-promoted translation in yeast. Nature 1998;392:516-520.

205. Gallie DR: A tale of two termini: a functional interaction between the termini of an mRNA is a prerequisite for efficient translation initiation. Genes Dev 1998;216:1-11.

206. Gallie DR: The cap and poly(A) tail function synergistically to regulate mRNA translation efficiency. Genes Dev 1991;5:2108-2116.

207. Doel MT, and Carey, N.H.: The translational capacity of deadenylated ovalbumin messenger RNA. Cell 1976;8:51-58.

208. Michel YM, Poncet, D., Prion, M., Kean, K.M., and Borman, A.M.: Cap-poly(A) synergy in mammalian cell-free extracts. Investigation of the requirements for poly(A) mediated stimulation of translation initiation. J Biol Chem 2000;275(41):32268-76.

209. Borman AM, Michel, Y.M., and Kean, K.M.: Biochemical characterization of cap-poly(A) synergy in rabbit reticulocyte lysates: the eIF4G-PABP interaction increases the functional affinity of eIF4E for the capped mRNA 5'-end. 2000.

210. Imataka H, Gradi, A., and Sonenberg, N.: A newly identified N-terminal amino acid sequence of human eIF4G binds poly(A) binding protein and functions in a poly(A)-dependent translation. EMBO J 1998;17:7480-89.

211. Tarun SZ, Jr., and Sachs, A.B.: Association of the yeast poly(A) tail binding protein with translation initiation factor eIF-4G. EMBO J 1996;15:7168-77.

212. Wells SE, Hillner, P., Vale, R., and Sachs, A.B.: Circularization of mRNA by eucaryotic translation initiation factors. Mol Cell 1998;2:135-40.

213. Morrissey JP, Deardorff, J.A., Hebron, C., and Sachs, AB.: Decapping of stabilized, polyadenylated mRNA in yeast *pab1* mutant. Yeast 1999;15:687-702.

214. Sachs A: Physical and functional interactions between the mRNA cap structure and the poly(A) tail, in Sonenberg N, Hershey, J. and Mathews M.B. (ed): Translational Control of Gene Expression. Cold Spring Harbor, Cold Spring Harbor Laboratory Press, 2000, pp 447-465.

215. Gale MJ, Tan, S-L., and Katze, M.G.: Translational control of viral gene expression in eukaryotes. Micro Mol Biol Rev 2000;64:239-80.

216. Garry RF, Bishop, J.M., Parker, S., Westbrook, K., Lewis, G., and Waite, M.R.F: $Na^+$ and $K^+$ concentrations and the regulation of protein synthesis in sindbis virus-infected chick cells. Virology 1979;96:108-20.

217. Inglis SC: Inhibition of host protein synthesis and degradation of cellular mRNAs during infection by influenza and Herpes simplex virus. Mol Cell Biol 1982;2:1644-48.

218. Fortes P, Beloso, A., and Ortin, J.: Influenza virus NS1 protein inhibits pre-mRNA splicing and blocks mRNA nucleocytoplasmic transport. EMBO 1994;13(704-12).

219. Katze MG, Detjen, B.M., Safer, B., and Krug, R.M.: Translational control by influenza virus: suppression of the kinase that phosphorylates the alpha subunit of initiation factor eIF-2 and selective translation of influenza viral-mRNAs. Mol Cell Biol 1986;6:1741-50.

220. Gale MJ, and Katze, M.G.: Molecular mechanisms of interferon resistance mediated by viral-detected inhibition of PKR, the interferon induced protein kinase. Pharmacol Ther 1998;78:29-46.

221. Salimans M, Goumans, H., Amesz,H., Benne, R. and Voorma, H.O.: Regulation of protein synthesis in eukaryotes. Mode of action of eRF, an eIF-2 recycling factor from rabbit reticulocytes involved in GDP/GTP exchange. Eur J Biochem 1984;145:91-98.

222. Clemens MJ: Regulation of eukaryotic protein synthesis by protein kinases that phosphorylate initiation factor eIF-2. Mol Biol Rep 1994a;19:201-10.

223. Clemens MJ, Laing, K.G., Jeffrey, I.W., Schofield, A., Sharp, T.V., Elia, A., Matys, V., James, M.A., and Tilleray, V.J.: Regulation of the interferon-inducible eIF-2$\alpha$ protein kinase by small RNAs. Biochemie 1994b;76(770-78).

224. Chang HW, Watson, J.C., and Jacobs, B.L.: The E3L gene of vaccinia virus encodes an inhibitor of the interferon-induced, double-stranded RNA-dependent protein kinase. PNAS 1992;89:4825-29.

225. Watson JC, Chang, H.W., and Jacobs, B.L.: Characterization of a vaccinia virus-encoded double-stranded RNA-binding protein that may be involved in inhibition of the double-stranded RNA-dependent protein kinase. Virology 1991;185:206-16.

226. Lu Y, Wambach, M., Katze, M.G., and Krug, R.M.: Binding of influenza virus NS1 protein to double-stranded RNA inhibits the activation of the protein kinase that phosphorylates the eIF-2 translation initiation factor. Virology 1995;214:222-28.

227. McMillan NA, Chun, R.F., Siderovski, D.P., Galabru, J., Toone, W.M.,, Samuel, C.E., Mak, T.W., Hovanaeeian, A.G., Jeang, K.T., and Williams, B.R.G: HIV-1 Tat directly interacts with the interferon-induced double-stranded RNA-dependent kinase, PKR. Virology 1995;213:413-24.

228. Hentze MW: eIF4G: a multipurpose ribosome adapter? Science 1997;275:500-501.

229. Keiper BD, Gan, W., and Rhoads, R.E.: Protein synthesis initiation factor 4G. Int J Biochem Cell Biol 1999;31:37-48.

230. Kaminski A, Belsham, G.J., and Jackson, R.J.: Translation of encephalomyocarditis virus RNA: parameters influencing the selection of internal initiation site. EMBO 1994;13:1673-81.

231. Kerekatte V, Keiper, B.D., Badorff, C., Cai, A., Knowlton, K.U., and Rhoads, R.E.: Cleavage of poly(A)-binding protein by coxsackievirus 2A protease in vitro and in vivo: another mechanism for host protein synthesis shutoff. J Virol 1999;73:709-717.

232. Borman AM, Deliat, F.G., and Kean, K.M.: Sequences within the poliovirus internal ribosome entry segment control viral RNA synthesis. EMBO 1994;13:3149-57.

233. Dominguez DI, Ryabova, L.A., Pooggin, M.M. Schmidt-Puchta, W., Futterer, J., and Hohn, T.: Ribosome shunting in cauliflower mosaic virus. Identification of an essential and sufficient structural element. J Biol Chem 1998;273:3669-78.

234. Ryabova LA, and Hohn, T.: Ribosome shunting in the cauliflower mosaic virus 35S RNA leader is a special case of reinitiation of translation function in plant and animal systems. Genes Dev 2000;14:817-29.

235. Kozak M: Effects of intercistronic length on the efficiency of reinitiation by eucaryotic ribosomes. Mol Cell Biol 1987b;7:3438-45.

236. Farabaugh PJ: Programmed translational frameshifting. Annu Rev Genet 1996;30:507-28.

237. Brierley I, Meredith, M.R., Bloys, A.J., and Hagervall, T.G.: Expression of a coronavirus ribosomal frameshift signal in *Escherichia coli*: influence of tRNA anticodon modification on frameshifting. J Mol Biol 1997;270:360-73.

238. Hua J, Mansell, E.A., and Patton, J.T.: Comparative analysis of the rotavirus NS53 gene: conservation of basic cysteine-rich regions in the protein and possible stem-structures in the RNA. Virology 1993;196:567-76.

239. McCrae MA, and Faulkner-Valle, G.P.: Molecular biology of rotaviruses I. Characterization of basic growth parameters and pattern of macromolecular synthesis. J Virol 1981;39:490-96.

240. Chizhikov V, and Patton J.T.: A four-nucleotide translation enhancer in the 3' terminal consensus sequence of the nonpolyadenylated mRNAs of rotavirus. RNA 2000;6:814-25.

241. Poncet D, Aponte, C., and Cohen, J.: Rotavirus protein NSP3 (NS34) is bound to the 3' end consensus sequence of viral mRNAs in infected cells. J Virol 1993;67(6):3159-65.

242. Tanguay RL, and Gallie, D.R.: Translational efficiency is regulated by the length of the 3' untranslated region. Mol Cell Biol 1996;16:146-56.

243. Park YW, and Katze, M.G.: Translational control by influenza virus. Identification of cis-acting sequences and trans-acting factors which may regulate selective viral mRNA translation. J Biol Chem 1995;270:28433-39.

244. Mattion NM, Cohen, J., Aponte, C., and Estes, M.K.: Characterization of an oligomerization domain and RNA-binding properties on rotavirus nonstructural protein NS34. Virology 1992;190:68-83.

245. Hua J, Chen, X., and Patton, J.: Deletion mapping of the rotavirus metalloprotein NS53 (NSP1): the conserved cysteine-rich region is essential for virus-specific RNA binding. J Virol 1994a;68(3990-4000).

246. Poncet D, Aponte, C., and Cohen, J.: Structure and function of rotavirus nonstructural protein NSP3. Arch Virol 1996;12(suppl):29-35.

247. Poncet D, Laurent, S., and Cohen, J.: Four nucleotides are the minimal requirement for RNA recognition by rotavirus non-structural protein NSP3. EMBO 1994;13(17):4165-73.

248. Vende P, Prion, M., Castagne, N., and Poncet, D.: Efficient translation of rotavirus mRNA requires simultaneous interaction of NSP3 with the eukaryotic translation initiation factor eIF4G and the mRNA 3' end. J Virol 2000;74(15):7064-71.

249. Piron M, Vende, P., Cohen, J., and Poncet, D.: Rotavirus RNA-binding protein NSP3 interacts with eIF4GI and evicts the poly(A) binding protein from eIF4F. EMBO J 1998;17:5811-21.

250. Groft CM, and Burley, S.K.: Recognition of eIF4G by rotavirus NSP3 reveals a basis for mRNA circularization. Mol Cell 2002;9:1273-83.

251. Padilla-Noriega L, Paniagua, O., and Guzman-Leon, S.: Rotavirus protein NSP3 shuts off host cell protein synthesis. Virology 2002;298:1-7.

252. Ericson BL, Graham, S.Y., Mason, B.B., and Estes, M.K.: Identification, synthesis, and modification of simian rotavirus SA11 polypeptides in infected cells. J Virol 1982;42:825-39.

253. Hua J, and Patton, J.: The carboxyl-half of the rotavirus nonstructural protein NS53 (NSP1) is not required for virus replication. Virology 1994b;196:567-76.

254. Gonzalez RA, Torres-Vega, M.A., Lopez, S., and Arias, C.F.: In vivo interactions among rotavirus nonstructural proteins. Arch Virol 1998;143:981-996.

255. Mitchell DB, and Both, G.W.: Conservation of a potential metal binding motif despite extensive sequence diversity in the rotavirus nonstructural protein NS53. Virology 1990;174:618-621.

256. Brotttier P, Nandi, P., Bremont, M., and Cohen, J.: Bovine rotavirus segment 5 protein expressed in the baculovirus system interacts with zinc and RNA. Journal of General Virology 1992;73:1931-38.

257. Taniguchi K, Kojima, K., Kobayashi, N., Urasawa, T. and Urasawa, S.: Structure and function of rotavirus NSP1. Arch Virol 1996a;12(suppl):53-58.

258. Evans RM, and Hollenberg, S.M.: Zinc fingers: guilt by association. Cell 1988;52:1-3.

259. Miller J, McLachlan, A.D., and Klug, A.: Repetitive zinc-binding domains in the protein transcription factor IIIA from *Xenopus* oocytes. EMBO J 1985;4:1609-14.

260. Taniguchi K, Kojima, K., and Urasawa, S.: Nondefective rotavirus mutants with an NSP1 gene which has a deletion of 500 nucleotides, including a cysteine-rich zinc finger motif-encoding region (nucleotide 156-248) or which has a nonsense codon at nucleotides 153-155. J Virol 1996b;70:4125-30.

261. Woode GN, Kelso, N.E., Simpson, T.F., Gaul, S.K., Evans, L.E., and Babiuk, L.: Antigenic relationships among some bovine rotaviruses serum neutralization and cross-protection in gnotobiotic calves. J Clin Microbiol 1983;18:358-64.

262. Bass DM, Baylor, M.R., Chen, C., Mackow, E.M., Bremont, M., and Greenberg H.B.: Liposome-mediated transfection of intact viral particles reveals that plasma membrane penetration determines permissivity of tissue culture cells in rotavirus. J Clin Invest 1992;90:2313-20.

263. Hardy ME, Woode, G.N., Xu, Z.C., and Gorziglia, M.: Comparative amino acid sequence analysis of VP4 for VP7 serotype 6 bovine rotavirus strain NCDV, B641, and UK. J Virol 1991;65:5535-38.

264. Hardy ME, Gorziglia, M., and Woode, G.N.,: Amino acid sequence analysis of bovine rotavirus B223 reveals a unique outer capsid protein VP4 and confirms a third bovine VP4 type. Virology 1992;191:291-300.

265. Bremont M, Charpilienne, A., Chabanne, D., and Cohen, J.: Nucleotide sequence and expression in *Escherichia coli* of the gene encoding the nonstructural protein NSP2 of bovine rotavirus. Virology 1987;161:138-44.

266. Estes MK, Mason..B., Crawford, S., and Cohen, J.: Cloning and nucleotide sequence of the simian rotavirus gene 6 that codes for the major inner capsid protein. Nucleic Acids Res 1984;12:1875-87.

267. Detjen BM, Walden, W.E., and Thach, R.E.: Translational specificity in reovirus-infected mouse fibroblasts. J Biol Chem 1982;257:9855-60.

268. Walden WE, and Thach, R.E.: Translational control of gene expression in a normal fibroblast. Characterization of a subclass of mRNAs with unusual kinetic properties. Biochemistry 1986;25:2033-41.

269. White MW, Degnin, C., Hill, J., and Morris, D.R.: Specific regulation by endogenous polyamines of translational initiation of S-adenosylmethionine decarboxylate mRNA in Swiss 3T3 fibroblasts. Biochem J 1990;268:657-60.

270. Lodish HF: Alpha and beta globin messenger ribonucleic acid. Different amounts and rates of initiation of translation. J Biol Chem 1971;246:7131-38.

271. Prasad BV, and Chiu, W.: Structure of Rotavirus. Curr Top Microbiol Immunol 1994;185:9-29.

272. Lawton JA, Estes, M.K., and Prasad, B.V.: Three-dimensional visualization of mRNA release from actively transcribing rotavirus particles [letter]. Nature Struct Biol 1997b;4:118-21.

273. Allen AM, and Desselberger, U.: Reassortment of human rotaviruses carrying rearranged genomes with bovine rotavirus. J Gen Virol 1985;66:2703-14.

274. Biryahwaho B, Hundley, F., and Desselberger, U.: Bovine rotavirus with rearranged genome reassorts with human rotavirus. Brief Report. Arch Virol 1987;96:257-64.

275. Kojima K, Taniguchi, K., Kawagishi-Kobayashi, M., Matsuno, S., and Urasawa, S.: Rearrangement generated in double genes, NSP1 and NSP3, of viable progenies from a human rotavirus strain. Virus Res 2000;67:163-71.

276. Pedley S, Hundley, F., Chrystie, I., McCrae, M.A., and Desselberger, U.: The genomes of rotaviruses isolated from chronically infected immunodeficient children. J Gen Virol 1984;65:1141-50.

277. Hundley F, Biryahwaho, B., Gow, M., and Desselberger, U.: Genome rearrangements of bovine rotavirus after serial passage at high multiplicity of infection. Virology 1985;143:88-103.

278. Walden WE, Godefroy-Colburn, T., and Thach, R.E.: The role of mRNA competition in regulating translation I. Demonstration of competition in vivo. J Biol Chem 1981;256:11739-46.

279. White MW, Kameji, T., Pegg, A.E., and Morris, D.R.: Increased efficiency of translation of ornithine decarboxylase mRNA in mitogen-activated lymphocytes. Eur J Biochem 1987;170:87-92.

280. Hundley F, McIntyre, M., Clark, B., Beards, G., Wood, D., Chrystie, I., and Desselberger, U.: Heterogeneity of genome rearrangements in rotaviruses isolated from a chronically infected immunodeficient child. J Virol 1987;61:3365-72.

281. Golini F, Thach, S.S., Birge, C.H., Safer, B., Merrick, W.C., and Thach, R.E.: Competition between cellular and viral mRNAs in vitro is regulated by a messenger discriminatory initiation factor. PNAS 1976;73:3040-44.

282. Ray A, Walden, W.E., Brendler, T., Zenger, V.E., and Thach, R.E.: Effect of medium hypertonicity on reovirus translation rates. An application of kinetic modeling in vivo. Biochemistry 1985;24:7525-32.

283. Brendler T, Godefroy-Colburn, T., Yu, S., and Thach, R.E.: The role of mRNA competition in regulating translation III. Comparison of in vitro and in vivo results. J Biol Chem 1981b;256:11755-61.

284. Brendler T, Godefroy-Colburn, T., Carlill, R.D., and Thach, R.E.: The role of mRNA competition in regulating translation II. Development of a quantitative in vitro assay. J Biol Chem 1981a;256:11747-54.

285. Godefroy-Colburn T, and Thach, R.E.: The role of mRNA competition in regulating translation. IV. Kinetic model. J Biol Chem 1981;256:11762-73.

286. Kozak M: An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic acids res 1987a;15:8125-48.

287. Kozak M: Compilation and analysis of sequences upstream from the translation start site in eukaryotic mRNAs. Nucleic acids res 1984;12:857-72.

288. Kozak M: Leader length and secondary structure modulate mRNA function under conditions of stress. Mol Cell Biol 1988;8:2737-44.

289. Kojima K, Taniguchi, K., and Kobayashi, N.: Species-specific and interspecies relatedness of NSP1 sequences in human, porcine, bovine, feline, and equine rotavirus strains. Arch Virol 1986;141:1-12.

290. Geballe AP, and Sachs, M.S.: Translational control by upstream open reading frames, in Sonenberg N, Hershey, J. and Mathews M.B. (ed): Translational Control of Gene Expression. Cold Spring Harbor, Cold Spring Harbor Laboratory, 2000, pp 595-614.

291. Gallie DR: Translational control of cellular and viral mRNAs. Plant Mol Biol 1996;32:145-58.

292. Gallie DR, and Kobayashi, M.: The role of the 3'-untranslated region of non-polyadenylated plant viral mRNAs in regulating translational efficiency. Gene 1994;142:159-65.

293. Hann LE, Webb, A.C., Cai, J.M., and Gehrke, L.: Identification of a competitive translation determinant in the 3' untranslated region of alfalfa mosaic virus coat protein mRNA. Mol Cell Biol 1997;17:2005-13.

294. Leathers V, Tanguay, R., Kobayashi, M., and Gallie, D.R.: A phylogenetically conserved sequence within viral 3' untranslated RNA pseudoknots regulates translation. Mol Cell Biol 1993;13:5331-47.

295. Zeyenko VV, Ryabova, L.A., Gallie, D.R., and Spirin, A.S.: Enhancing effect of the 3'-untranslated region of tobacco mosaic virus RNA on protein synthesis in vitro. FEBS lett 1994;354:271-73.

296. Patton JT, Taraporewala, Z., Chen, D., Chizhikov, V., Jones, M., Elhelu, A., Collins, M., Kearney, K., Wagner, M., Hoshino, Y., and Gouvea, V: Effect of intragenic rearrangement and changes in the 3' consensus sequence on NSP1 expression and rotavirus replication. J Virol 2001;75:2076-86.

297. Mitzel DN, Weisend, C.M., White, M.W., and Hardy, M.E.: Translation regulation of rotavirus gene expression. J Gen Virol 2003;84:1-9.

298. Tang B, Gilbert, J.M., Matsui, S.M., and Greenberg, H.B.: Comparison of rotavirus gene 6 from different species by sequence analysis and localization of subgroup-specific epitopes using site-directed mutagenesis. Virology 1997;237:89-96.

299. Green KY, Hoshino, Y., and Ikegami, N.: Sequence analysis of the gene encoding the serotype-specific glycoprotein (VP7) of two new human rotavirus serotypes. Virology 1989;168:429-33.

300. Garfinkel MS, and Katze, M.G.: Translational control by influenza virus. Selective translation is mediated by sequences within the viral mRNA 5'-untranslated region. J Biol Chem 1993;268:22223-26.

301. Aragon T, DeLa Luna, S., Novoa, I., Carrasco, L., Ortin, J., and Nieto, A.: Eukaryotic translation initiation factor 4GI is a cellular target for NS1 protein, a translational activator of influenza virus. Mol Cell Biol 2000;20:6259-68.

302. Yang J, Chennathukuzhi, V., Miki, K., O'Brien, D., and Hecht, N.B.: Mouse testis brain RNA-binding protein/translin selectively binds to the messenger RNA of the fibrous sheath protein glyceraldehyde 3-phosphate dehydrogenase-S and supresses its translation in vitro. Biol Reprod 2003;68(853-59).

303. Xu Y-H, and Grabowski, G.A.: Molecular cloning and characterization of a translational inhibitory protein that binds to coding sequences of human acid β-glucosidase and other mRNAs. Mol Gen Metab 1999;68:441-54.

304. Enami K, Sato, T.A., Nakada, S., and Enami, M.: Influenza virus NS1 protein stimulates translation of the M1 protein. J Virol 1994;68:1432-37.

305. Ray BK, Brendler, T.G., Adya, S., Daniels-McQueen, S., Miller, W.C., Hershey, J.W.B., Grifo, J.A., Merrick, W.C., and Thach, R.E.: Role of mRNA competition in regulating translation: further characterization of mRNA discriminatory initiation factors. PNAS 1983;80:663-67.

306. Hesketh J: Translation and the cytoskeleton: a mechanism for targeted protein synthesis. Mol Biol Rep 1994;19:233-43.

307. Bernstein E, Denli, A.M., and Hannon, G.J.: The rest is silence. RNA 2001;7:1509-21.