Translational regulation of rotavirus gene expression

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Rotavirus mRNAs are transcribed from 11 genomic dsRNA segments within a subviral particle. The mRNAs are extruded into the cytoplasm where they serve as mRNA for protein synthesis and as templates for packaging and replication into dsRNA. The molecular steps in the replication pathway that regulate the levels of viral gene expression are not well defined. We have investigated potential mechanisms of regulation of rotavirus gene expression by functional evaluation of two differentially expressed viral mRNAs. NSP1 (gene 5) and VP6 (gene 6) are expressed early in infection, and VP6 is expressed in excess over NSP1. We formulated the hypothesis that the amounts of NSP1 and VP6 were regulated by the translational efficiencies of the respective mRNAs. We measured the levels of gene 5 and gene 6 mRNA and showed that they were not significantly different, and protein analysis indicated no difference in stability of NSP1 compared with VP6. Polyrribosome analysis showed that the majority of gene 6 mRNA was present on large polysomes. In contrast, sedimentation of more than half of the gene 5 mRNA was subpolysomal. The change in distribution of gene 5 mRNA in polysome gradients in response to treatment with low concentrations of cycloheximide suggested that gene 5 is a poor translation initiation template compared with gene 6 mRNA. These data define a regulatory mechanism for the difference in amounts of VP6 and NSP1 and provide evidence for post-transcriptional control of rotavirus gene expression mediated by the translational efficiency of individual viral mRNAs.

INTRODUCTION

The rotavirus genome consists of 11 segments of dsRNA, which are packaged into a structurally complex triple-layered particle (Prasad & Chiu, 1994). Viral mRNAs transcribed from dsRNA have a 5’-terminal cap structure m7GpppG(m)Gpy and are not polyadenylated (Imai et al., 1983; McCrae & McCorquodale, 1983). Nine of the 11 dsRNA segments are monocistronic, the exceptions being gene 11, which encodes NSP5 and NSP6, and the VP7 gene, which has an alternative initiation codon (Estes & Cohen, 1989; Mattion et al., 1991). The open reading frame of each gene is flanked by 5’ and 3’ untranslated regions (UTRs) that vary in length and sequence between each segment, although short consensus sequences at both the 5’ and 3’ ends are conserved (Patton, 1995). Rotavirus mRNAs transcribed within subviral particles are extruded into the cytoplasm through pores in the capsid layers located at the icosahedral fivefold axes (Lawton et al., 1997). 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 & McCrae (1989) measured the levels of viral transcripts and encoded proteins and showed that there was quantitative as well as temporal control of rotavirus gene expression. 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. Furthermore, 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 non-structural protein NSP3 and the cellular translation initiation factor eIF4GI (Piron et al., 1998). NSP3 is a sequence-specific RNA-binding protein that binds to the last four to five conserved nucleotides of the 3’ end of viral mRNAs (Poncet et al., 1994). 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 most likely promotes circularization of viral mRNAs to enhance translation, as
previously proposed (Piron et al., 1998; Vende et al., 2000). At the RNA level, a four-nucleotide translation enhancer element in the 3'-terminal consensus sequence of rotavirus mRNAs has been reported (Chizhikov & Patton, 2000). 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 have investigated potential mechanisms of post-transcriptional regulation of rotavirus gene expression by analysing the translational efficiencies of gene 5 and gene 6 mRNAs. These two genes were chosen as models for analysis of rotavirus gene regulation because: (i) both are expressed early in infection; (ii) VP6 is expressed at higher levels than NSP1; and (iii) the mRNAs and ORFs are similar in size, thus minimizing potential variations in expression due to mRNA length. Gene 5 encodes non-structural protein NSP1. The function of NSP1 in infected cells is not clear, but NSP1 appears to be non-essential for replication, because viruses with gene 5 rearrangements that do not synthesize NSP1 have been isolated (Allen & Desselberger, 1985; Biryahwaho et al., 1987; Kojima et al., 2000; Pedley et al., 1984; Taniguchi et al., 1996). Gene 6 encodes the major inner capsid protein, VP6, which is the subgroup-specific antigen and is required for viral transcriptase activity (Estes & Cohen, 1989). We examined mRNA accumulation, protein stability and polyribosome distribution of genes 5 and 6 mRNA in rotavirus-infected cells. Our data suggest that the difference in the amounts of NSP1 and VP6 is accounted for by a difference in the translational efficiencies of the mRNA.

### METHODS

**Virus and cells.** Isolation and characterization of bovine rotavirus strain B641 has been described (Woode et al., 1983). B641 was cultured in MA104 cells maintained in M199 medium (Irving Scientific) in the presence of 5% foetal bovine serum (FBS; Atlanta Biologicals). Cells were infected at approximately 90 % confluency with 5×10⁵ TCID₅₀ of the virus. Cells were infected at an m.o.i. of 0.1 for stock virus preparation and an m.o.i. of 5–10 for RNA or protein analysis. The virus was activated by treatment for 30 min at 37°C. Activated virus was adsorbed to cell monolayers either at 4 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 (Bass et al., 1992). Cultures were shifted to 37°C after a 1–5 h 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 (Hardy et al., 1991, 1992). Purified mRNA was reverse-transcribed for 3 h with avian myeloblastosis virus reverse transcriptase (Promega) primed with the oligonucleotide 5'-CACGGATCGGTCACATTTTGCAGGGAGTCTTG-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) by 30 cycles of PCR with the above primer and the positive-sense primer 5'-CAGGGATCCAGGTTTGTCCAGGATCTCTG-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 min, 50°C for 45 s and 72°C for 2 min. The resultant 1.5 kb PCR product was cloned into pBluescript KS+ (Stratagene). The nucleotide sequence was determined on an ABI 310 Genetic Analyser 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 (Bremont et al., 1987).

**Metabolic labelling of rotavirus proteins.** Viral proteins synthesized in infected cells were labelled as previously described, with modifications (Ericson et al., 1982). MA104 cells were infected at an m.o.i. of 10 in medium lacking methionine and cysteine and containing 5 % FBS. At 4 h post-infection (p.i.), the medium was replaced with medium without FBS and containing 10 μg actinomycin D ml⁻¹ and 40 μCi TRAN³⁵S-label ml⁻¹ (ICN). Cultures were harvested at the indicated times by gentle rocking in RIPA buffer (130 mM NaCl, 1 % sodium deoxycholate, 1 % Triton X-100, 0-1 % SDS, 10 mM Tris/HCl, pH 7.2, 1 % Trasylol). Labelled proteins were resolved by SDS-PAGE and visualized by autoradiography.

Pulse-chase labelling was performed as previously described (Ericson et al., 1982) and as outlined above, except that following a 30 min pulse with 40 μCi TRAN³⁵S-label ml⁻¹, cultures were chased with medium containing 400 μCi unlabelled methionine and cysteine and 100 μg cycloheximide ml⁻¹. Cultures were chased for the indicated times, harvested in RIPA buffer and radio-labelled proteins were analysed by SDS-PAGE. The identity of the band in MA104 cells infected with rotavirus under these conditions has been confirmed by reactivity with an anti-NSP1 monoclonal antibody. Radioactive protein bands were quantified on a Bio-Rad Molecular Imager F-X with Quantity One software.

**RNA extraction and Northern blot hybridization.** Total RNA was harvested from mock-infected or B641-infected cells with Trizol (Invitrogen–Life Technologies) 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. Radio labelled probes were prepared by nick-translation of specific gene fragments, which were digested and purified from plasmid vectors. The plasmid containing SA11 gene 6 in the pSP65 vector (Promega) was a gift from M. K. Estes (Baylor College of Medicine, Houston, Texas, USA) (Estes et al., 1984). This plasmid was transformed into E. coli DH5α cells and mid-scale plasmid purifications were performed by standard methods (Sambrook & Russell, 2001). The specific activity of the probes was determined by liquid scintillation counting and care was taken to ensure that 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 Bio-Rad Molecular Imager F-X with Quantity One software.

**Polyribosome analysis.** All polyribosome analyses were performed several times and representative data are shown. Cytoplasmic extract preparation and polyribosome analysis were performed and interpreted as previously described (Detjen et al., 1982; Kaspar et al., 1992; Lodish, 1971; Walden & Thach, 1986; White et al., 1990). MA104 cells were mock-infected or infected with B641 at an m.o.i. of 5–10. At the indicated times p.i., the cells were treated with 100 μg cycloheximide ml⁻¹ to arrest ribosome transit. Cells were collected from the dishes with a 1× trypsin solution containing 100 μg cycloheximide ml⁻¹. Cells were swollen in low-salt buffer (20 mM Tris/HCl,
pH 7-4, 10 mM NaCl, 3 mM MgCl₂) and lysed with low-salt buffer containing 1-2 % Triton N-101 and 200 mM sucrose and nine strokes of a Dounce homogenizer. Cell nuclei were removed by brief centrifugation and cell lysates were layered onto 0-5–1-5 M sucrose gradients prepared in low-salt buffer. The gradients were centrifuged for 58 min at 159 000 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 µ fractions with 1:1 phenol/chloroform, then with chloroform and finally precipitated with 250 mM NaCl, 20 µg glycogen ml⁻¹ and ethanol. The RNA from each fraction was electrophoresed through 1-2 % agarose/6 % formaldehyde gels and subjected to Northern blot hybridization analysis as described above.

RESULTS

Synthesis of NSP1 and VP6 in B641-infected cells

NSP1 and structural protein VP6 are expressed early in infection (Ericson et al., 1982). Johnson & McCrae (1989) reported that in BSC-1 cells infected with bovine rotavirus strain UK (Compton), VP6 was present in approximately 25-fold molar excess over NSP1. We analysed 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 TRAN³⁵S-label. The cultures were harvested 4 h p.i. and labelled proteins were separated by SDS-PAGE and visualized by autoradiography. Fig. 1 shows the typical pattern of rotavirus protein synthesis. Quantification of ~50 kDa NSP1 and ~44 kDa VP6 by densitometry showed VP6 present in at least 10-fold excess over NSP1. This is most 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 VP6. Analysis of the levels of VP6 and NSP1 at 6 and 8 h p.i. indicated the relative ratios of these two proteins at later times in the infection remained at 10-fold (6 h) or increased to 15-fold (8 h) (data not shown). These data suggest that differential regulation of synthesis of VP6 and NSP1 was maintained throughout the infectious cycle.

A pulse-chase analysis was performed to determine whether differences in protein stability accounted for the excess of VP6 over NSP1. Pulse-labelling at 4 h p.i. followed by chase times up to 3 h indicated no significant difference in the stability of NSP1 and VP6, with both proteins displaying half-lives of approximately 45 min (Fig. 2). These data are in agreement with those reported by Hundley et al. (1985) and suggest expression of these two proteins must be regulated at the RNA level.

Messenger RNA levels for genes 5 and 6 at 4 h p.i. 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 whether 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 (Bass et al., 1992). 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 4 h and accumulation of mRNA for genes 5 and 6 was analysed by Northern blot hybridization to gene-specific probes comparable in length and specific activity. The data in Fig. 3 show that the levels of each mRNA up to and including 4 h p.i. were not significantly different. Quantification of the radioactive signals indicated that the magnitude of the difference between the two mRNAs was less than twofold. These data, taken together with the protein analysis described above, suggest that 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 distribution can determine whether an mRNA is efficiently translated and if the...
efficiency of translation is regulated at the initiation step
(Lodish, 1971). We first evaluated the distribution of gene
5 mRNA in polysome gradients to determine whether
the low expression of NSP1 compared with VP6 was due
to differences in the translational efficiencies of the two
mRNAs. B641-infected MA104 cells were harvested 4 h p.i.
and the lysate was centrifuged through 0.5–1.5 M linear
sucrose gradients. RNA was harvested in RIPA buffer and labelled 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 with Quantity One software and plotted as decrease
in signal over time.

Fig. 2. Pulse-chase labelling of B641 rotavirus-infected cells.
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 TRAN35 S-label ml⁻¹, then chased for indicated
times with medium containing 400 × methionine and cysteine
and 100 µg cycloheximide ml⁻¹. Cultures were harvested in
RIPA buffer and labelled 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 with Quantity One software and plotted as decrease
in signal over time.

Fig. 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 h. The RNA was
electrophoresed on 1-2 % agarose/formaldehyde gels, trans-
ferred to nylon membranes and hybridized with 32P-labelled
probes specific for gene 5, gene 6, or actin as a loading con-
trol. Signals were quantified with a Bio-Rad Molecular
Imager FX with Quantity One software.

Fig. 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
159 000 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, sepa-
rated on a 1-2 % agarose/formaldehyde gel and transferred to
nylon membrane. The blot was probed with a 32P-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.
ribonucleoprotein particles (mRNPs) and pre-initiation complexes. Approximately 55% of gene 5 mRNA was found in subpolysomal fractions. We analysed the distribution of gene 6 mRNA for comparison and control (see Fig. 6, left panel). In contrast to gene 5 mRNA, the majority of gene 6 mRNA was associated with large polysomes. Quantification of the hybridization signal indicated that ~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 4 h p.i.

The rotavirus replication cycle in monkey kidney cells in culture is approximately 8 h (Johnson & McCrae, 1989). We performed additional polysome analyses of both genes as described above at 2 and 8 h p.i. to determine whether the observed sedimentation was altered at earlier and later times (Fig. 5). At 2 h p.i., ~67% of gene 5 mRNA (Fig. 5A) and 52% of gene 6 (data not shown) was present in subpolysomal fractions. At 8 h p.i., ~89% of the gene 5 mRNA (Fig. 5B) and 69% of gene 6 mRNA (data not shown) was subpolysomal. This predominately subpolysomal sedimentation of gene 5 and 6 mRNAs at 8 h p.i. 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 that 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 Figs 4 and 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 mRNA in subpolysomal fractions of the gradient may be untranslatable and bound in mRNPs. The presence of rotavirus mRNA in untranslated mRNPs is a formal possibility, given that viral mRNAs must also serve as

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**Fig. 5.** Distribution of gene 5 mRNA in polyribosome gradients at 2 and 8 h p.i. 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 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.

**Fig. 6.** Distribution of gene 5 and gene 6 mRNA in response to infection in the presence 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 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 hybridizations 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. Percentages of mRNA in polysomal and subpolysomal fractions are shown below the blots. Data shown are representative of multiple experiments.
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 cycloheximide ml⁻¹ for 45 min prior to harvest. Low concentrations of cycloheximide (compare 0.5 μg ml⁻¹ in this experiment with 100 μg ml⁻¹ 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 (Lodish, 1971; Walden et al., 1981; White et al., 1987). 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 Fig. 6 suggested that 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 Fig. 4, as ~42% of the mRNA displayed subpolysomal sedimentation and ~58% of the mRNA was found associated with polysomes (Fig. 6, left panel). Treatment of infected cells with 0.5 μg cycloheximide ml⁻¹ resulted in a shift in the distribution of the mRNA toward the heavier fractions of the gradient containing large polysomes (Fig. 6, right panel). Quantification of the hybridization signals indicated that ~80% of gene 5 mRNA was found in polysome fractions in the presence of cycloheximide. Northern blot hybridization to detect gene 6 mRNA also demonstrated a detectable shift (~72% to ~93%), but not nearly as dramatic as that 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 analysing mRNA accumulation, protein stability and polyribosome distributions of two differentially expressed genes encoding NSP1 and VP6. The data showed that the mRNA encoding NSP1 was inefficiently translated. A bimodal sedimentation and the response of gene 5 mRNA to low concentrations of cycloheximide indicated that expression of NSP1 was 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 h) or predominately subpolysomal (8 h), suggesting that regulation of NSP1 expression was maintained throughout the replication cycle. This distribution of gene 5 mRNA at the 8 h 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 supported 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 (Hua & Patton, 1994; Hua et al., 1993). The low level of expression of NSP1 persists throughout the replication cycle (Ericson et al., 1982). The number of functional molecules of NSP1 in infected cells is most 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 (Biryahwaho et al., 1987; Hundley et al., 1985, 1987; Pedley et al., 1984; Taniguchi et al., 1996). 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 with their wild-type counterparts.

In contrast to a regulatory function for NSP1, VP6 forms the major inner capsid layer of the mature virus particle. The mature virion contains 260 trimers of VP6 (Prasad & Chiu, 1994). If a single infected cell releases 50–60 p.f.u. (Johnson & McCrae, 1989), 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 p.f.u. ratio of 1 and the particle to p.f.u. ratio for rotavirus has been reported to be 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 have 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 (Detjen et al., 1982; Golini et al., 1976; Lawson et al., 1988; Ray et al., 1983; Walden et al., 1981). Walden et al. (1981) reported hierarchical translation efficiency of mRNAs transcribed from reovirus gene segments. 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 that the competitive ability of a mRNA was determined by its ability to efficiently recruit translation initiation factors (Brendler et al., 1981a, b; Godefroy-Colburn & Thach, 1981; Walden et al., 1981). 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 (Kozak, 1984, 1987, 1988). 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′UTRs 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 at 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 (Bremont et al., 1987), UK (Hua & Patton, 1994) and A44 (Kojima et al., 1996) that could negatively affect the efficiency of translation of this mRNA. Translation 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 (reviewed in Geballe & Sachs, 2000). Evidence continues to accumulate that implicates the 3′UTR in translational control of gene expression in both cellular and viral mRNAs (Gallie, 1996; Gallie & Kobayashi, 1994; Hann et al., 1997; Leathers et al., 1993; Tanguay & Gallie, 1996; Zeyenko et al., 1994). Tanguay & Gallie (1996) reported that a longer 3′UTR on a mRNA lacking a poly(A) tail increased the translational efficiency of a luciferase reporter mRNA and this effect was sequence-independent and gene-independent. Rotavirus mRNAs do not have poly(A) 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 with 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 (Tanguay & Gallie, 1996). It was suggested that following termination and dissociation of the 80S ribosome, the 40S ribosomal subunit continues to transit the 3′UTR. Therefore, mRNAs 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 (GACC) present at the 3′ termini of rotavirus genes that enhanced expression of a luciferase reporter gene when RNA encoding this sequence was transfected into rotavirus-infected cells (Chizhikov & Patton, 2000). These four nucleotides, when present at the 3′ terminus of rotavirus mRNA, bind NSP3 (Poncet et al., 1993), and it was proposed that variation in this sequence could negatively affect viral protein expression by altering binding of NSP3 (Patton et al., 2001). 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.

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nucleotides, including a cysteine-rich zinc finger motif-encoding region (nucleotides 156 to 248), or which has a nonsense codon at nucleotides 153–155. J Virol 70, 4125–4130.


