RNA (2000), *6*:814–825. Cambridge University Press. Printed in the USA.

A four-nucleotide translation enhancer in the 3′-terminal consensus sequence of the nonpolyadenylated mRNAs of rotavirus

VLADIMIR CHIZHIKOV and JOHN T. PATTON

Laboratory of Infectious Diseases, National Institutes of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892, USA

ABSTRACT

The 5′ cap and poly(A) tail of eukaryotic mRNAs work synergistically to enhance translation through a process that requires interaction of the cap-associated eukaryotic initiation factor, eIF-4G, and the poly(A)-binding protein, PABP. Because the mRNAs of rotavirus, and other members of the *Reoviridae*, contain caps but lack poly(A) tails, their translation may be enhanced through a unique mechanism. To identify translation-enhancement elements in the viral mRNAs that stimulate translation in vivo, chimeric RNAs were prepared that contained an open reading frame for luciferase and the 5′ and 3′ untranslated regions (UTRs) of a rotavirus mRNA or of a nonviral mRNA. Transfection of the chimeric RNAs into rotavirus-infected cells showed that the viral 3′ UTR contained a translation-enhancement element that promoted gene expression. The element did not enhance gene expression in uninfected cells and did not affect the stability of the RNAs. Mutagenesis showed that the conserved sequence GACC located at the 3′ end of rotavirus mRNAs operated as an enhancement element. The 3′-GACC element stimulated protein expression independently of the sequence of the 5′ UTR, although efficient expression required the RNA to contain a cap. The results indicate that the expression of viral proteins in rotavirus-infected cells is specifically up-regulated by the activity of a novel 4-nt 3′ translation enhancer (TE) common to the 11 nonpolyadenylated mRNAs of the virus. The 4-nt sequence of the rotavirus 3′ TE represents by far the shortest of any of the sequence enhancers known to stimulate translation.

Keywords: gene expression; nonpolyadenylated mRNAs; rotavirus; translation enhancer

INTRODUCTION

Biochemical and genetic evidence indicates that the 5′ cap and poly(A) tail of eukaryotic mRNAs work synergistically to stimulate translation initiation (reviewed in Gallie, 1998). Communication between the cap and poly(A) tail is mediated by interactions involving the poly(A)-binding protein, PABP, and the cap-associated eukaryotic initiation factor, eIF-4G (Tarun & Sachs, 1996; Le et al., 1997; Imataka et al., 1998), and results in the circularization of mRNAs contained in polysomes (Wells et al., 1998). The RNA–protein and protein–protein interactions that direct circularization are believed to enhance translation by stabilizing the binding of initiation factors to the mRNA and by promoting 3′ → 5′ recycling of ribosomes (Gallie, 1991, 1998; Jacobson, 1996; Preiss & Hentze, 1998). A number of mRNAs have been described that, as a result of a lack of a 5′ cap and/or poly(A) tail, have evolved novel alternative mechanisms for achieving efficient translation. For example, the capped but nonpolyadenylated mRNAs of alfalfa mosaic virus (AMV) and tobacco mosaic virus (TMV) both contain determinants in their 3′ untranslated region (UTR) that operate as translation enhancers (TE) and that appear to function as surrogates of the poly(A) tail (Gallie & Walbot, 1990; Hann et al., 1997). In addition, the noncapped and nonpolyadenylated mRNAs of satellite necrosis virus (STNV) and barley yellow dwarf virus (BYDV) also contain 3′ sequences that enhance translation (Meulewaeter et al., 1998; Wang et al., 1999).

Rotaviruses are members of the *Reoviridae*, a large family of viruses that contain segmented genomes consisting of double-stranded (ds)RNAs (Estes, 1996). The mRNAs made by these viruses are capped but nonpolyadenylated (Imai et al., 1983; McCrae & McCorquodale, 1983) and serve two functions in infected cells: to direct the synthesis of viral proteins and as templates for the synthesis of minus-strand RNA to form dsRNA (Chen et al., 1994). The 11 mRNAs of rotavirus

Reprint requests to: John T. Patton, Laboratory of Infectious Diseases, National Institutes of Allergy and Infectious Diseases, National Institutes of Health, 7 Center Drive, MSC 0720, Room 117, Bethesda, Maryland 20892, USA; e-mail: jpatton@atlas.niaid.nih.gov.

are of 0.7–3.3 kb with 5′ UTRs of 9–49 b and 3′ UTRs of 17–182 b (Estes, 1996). Except for the mRNA of segment 11, which is dicistronic, the mRNAs of rotavirus are monocistronic (Mitchell & Both, 1988). The only conserved regions of the mRNAs are located at the 5′ and 3′ termini, which have the sequences GGC(U/A)$_{6–9}$ and UGUGACC, respectively (Desselberger & McCrae, 1994). Differences in efficiencies of transcription and translation of the viral mRNAs result in the accumulation of different levels of rotavirus proteins in the infected cell (Johnson & McCrae, 1989). The mechanism by which efficient translation of the viral mRNAs is achieved is not known, but given their lack of poly(A) tails, can be expected to differ from that of polyadenylated mRNAs.

Previous studies have provided evidence that suggests that the rotavirus nonstructural protein, NSP3, may play a role in the efficient translation of viral mRNAs (Piron et al., 1998). NSP3 is a 34-kDa protein that forms dimers that can specifically bind to the last 4 nt of the conserved 3′-terminal sequence of viral mRNAs (GACC) (Poncet et al., 1993, 1994; Piron et al., 1999). NSP3 also binds to eIF-4GI and, in doing so, destabilizes the interaction of PABP with eIF-4F, properties that Piron et al. (1998) have suggested may lead to an inhibition of cellular mRNA translation. Although direct evidence is lacking, the ability of NSP3 to bind to both the 3′ end of the mRNA and the cap-associated eIF-4GI may allow the formation of circular polysomes that could direct the efficient synthesis of viral protein (Piron et al., 1998).

The location and properties of *cis*-acting elements in rotavirus mRNAs that enhance translation have not been described. To identify such elements, we produced chimeric RNAs that contained the open reading frame (ORF) for luciferase and the 5′ and 3′ UTRs of the rotavirus VP6 mRNA (gene 6) or of nonviral mRNAs. Transfection of the chimeric RNAs into rotavirus-infected cells revealed that the viral 3′ UTR contained one or more elements that significantly enhanced luciferase expression. Mutagenesis established that only a single element was present in the 3′ UTR and that the element was located in the conserved 3′-terminal sequence of rotavirus mRNAs and had the sequence GACC. The 3′ TE did not stimulate translation of RNAs in uninfected cells or of RNAs that lacked caps, and the activity of the 3′ TE was not influenced by the sequence of the 5′ UTR. These data indicate that the expression of viral proteins in rotavirus-infected cells is specifically up-regulated by the activity of a novel 4-nt 3′ TE common to all the nonpolyadenylated mRNAs of the virus.

RESULTS

Enhancement of gene expression in rotavirus-infected cells

To investigate whether the 5′ and 3′ UTRs of rotavirus mRNAs contain elements that promote gene expression in infected cells, we produced an analog of the gene 6 mRNA of simian rotavirus SA11 (Fig. 1). The analog,

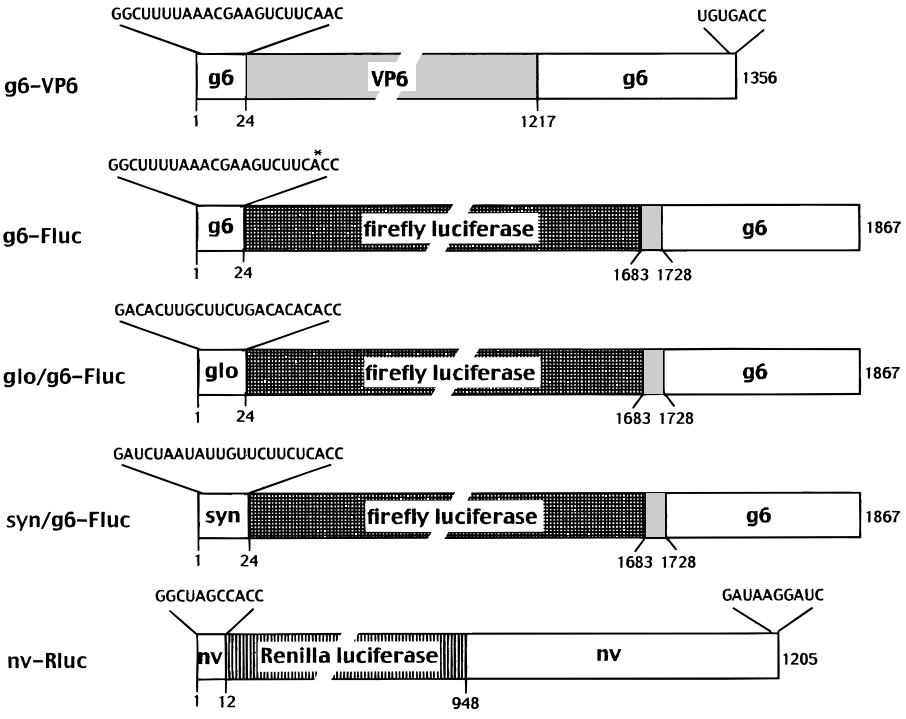

FIGURE 1. Chimeric RNAs containing an ORF for luciferase. Rotavirus gene 6 mRNA (g6-VP6) encodes VP6 and has 5′ and 3′ UTRs of 23 and 139 bases, respectively. The chimeric RNA, g6-Fluc, is an analog of the gene 6 mRNA, and contains the ORF for firefly luciferase instead of the ORF for VP6. The UTRs of g6-Fluc RNA are the same as that of g6-VP6 RNA, except that nt 23 of the 5′ UTR is a C instead of an A (*). The first 19 bases of the 5′ UTRs of the glo/g6-Fluc and syn/g6-Fluc RNAs represent the first 19 bases of the 5′ UTR of β-globin mRNA and of a random sequence, respectively. Otherwise, the glo/g6-Fluc and syn/g6 Fluc RNAs are identical to g6-Fluc RNA. Region 1683–1728 of the Fluc chimeric RNAs represents the last 45 nt of the VP6 ORF. Note that none of the mRNAs are polyadenylated, that the last 4 nt of the 5′ UTRs of all the chimeras are CACC, and that the last 5 nt are UGACC for the g6-Fluc, glo-g6-Fluc, and syn-g6-Flu chimeras, and is GGAUC for the nv-Fluc RNA. The nv-Rluc RNA contains the ORF for *Renilla* luciferase instead of firefly luciferase.

g6-Fluc RNA, is a chimera that contains the 23-nt 5′ UTR and 139-nt 3′ UTR of gene 6 and that, instead of the ORF for VP6, contains the ORF for firefly luciferase. Capped g6-Fluc RNA was cotransfected with capped nonviral RNA encoding *Renilla* luciferase (nv-Rluc RNA) (Fig. 1) into mock-infected and rotavirus-infected cells, and the expression of firefly and *Renilla* luciferases determined at 2-h intervals from 3 to 11 h postinfection. The results showed that firefly and *Renilla* luciferases were produced in mock-infected cells at low levels and at approximately the same levels during this time (Fig. 2A). Similarly, the expression of both types of luciferases was low at 3 h postinfection in infected cells, but beginning at 5 h pi and progressing to 11 h postinfection, a continuous increase was observed in the production of firefly luciferase. By 11 h postinfection, the level of firefly luciferase was 75-fold greater in infected cells than in uninfected cells, indicating that viral infection caused a significant increase in the expression of the reporter enzyme (Fig. 2A). The kinetics of firefly luciferase expression paralleled that of the expression of viral proteins in infected cells, which became readily detectable at 5 h postinfection and increased in levels through 11 h postinfection (Fig. 2B). The production of *Renilla* luciferase from the nv-Rluc RNA also increased from 5 to 11 h postinfection in infected cells, and reached a level by 11 h postinfection that was 15-fold greater than that observed in uninfected cells. However, the fact that viral infection resulted in a fivefold increase in the level of firefly luciferase beyond that of *Renilla* luciferase at 11 h postinfection suggested that the viral-specific 5′ and 3′ UTRs of the g6-Fluc RNA contained one or more elements that enhanced gene expression, but only in the infected cell.

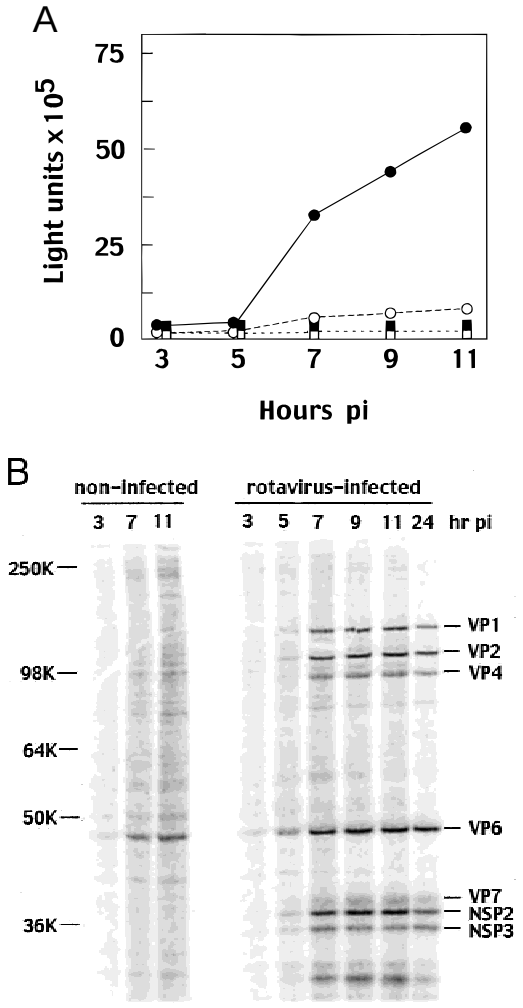

FIGURE 2. Importance of the viral UTRs to protein expression in noninfected and infected cells. **A**: MA104 cells were mock-infected (□, ■) or rotavirus-infected (○, ●) and, at 1 h postinfection, transfected with g6-Fluc RNA (●, ■) and nv-Rluc RNA (○, □). At 2-h intervals beginning at 3 h postinfection, the cells were harvested and the levels of firefly and *Renilla* luciferases per milligram of lysate protein were determined. **B**: Non-infected and rotavirus-infected MA104 cells were maintained continuously in the presence of ^{35}S-amino acids and were harvested at 2-h intervals from 3 to 11 h postinfection. Cellular lysates were analyzed by electrophoresis on a 10% polyacrylamide gel containing SDS and by autoradiography. The location of viral structural (VP) and nonstructural (NS) proteins and molecular weight markers are indicated.

Enhancement signal in the 3′ UTR of rotavirus mRNAs

The absence of a 3′ poly(A) tail on rotavirus mRNAs led us to examine the possibility that an element located in the 3′ UTR could operate as a surrogate for the poly(A) tail and function in the infected cell to specifically enhance the translation of viral mRNAs. To test for a 3′ TE, deletions of increasing length (3–139 nt) were made from the 3′ end of the g6-Fluc RNA. The modified RNAs containing 5′ caps were then cotransfected with capped nv-Rluc RNA into rotavirus-infected cells and the expression of firefly and *Renilla* luciferases determined independently. The values obtained for the expression of firefly luciferase were then normalized against the expression of *Renilla* luciferase, to minimize possible variations in transfection efficiencies. The results showed that deletion of as few as three bases from the 3′ end of g6-Fluc RNA caused a three- to fourfold reduction in the expression of firefly luciferase (Fig. 3A). Deletion of more than three bases, even up to the point that the entire 3′ UTR of the chimera was removed, caused no additional reduction in the expression of firefly luciferase. Thus, the last three bases at the 3′ end of the g6-Fluc RNA were required for efficient expression of luciferase. Indeed, mutagenesis of the last two bases at the 3′ end of the RNA from CC to GG caused a reduction in luciferase expression that was similar to that seen when the last three bases were deleted (Fig. 3A), indicating that the last two residues of the RNA were essential for efficient translation of the RNA and were part of the 3′ TE.

Although transfection of g6-Fluc RNA led to the expression of less firefly luciferase in mock-infected cells

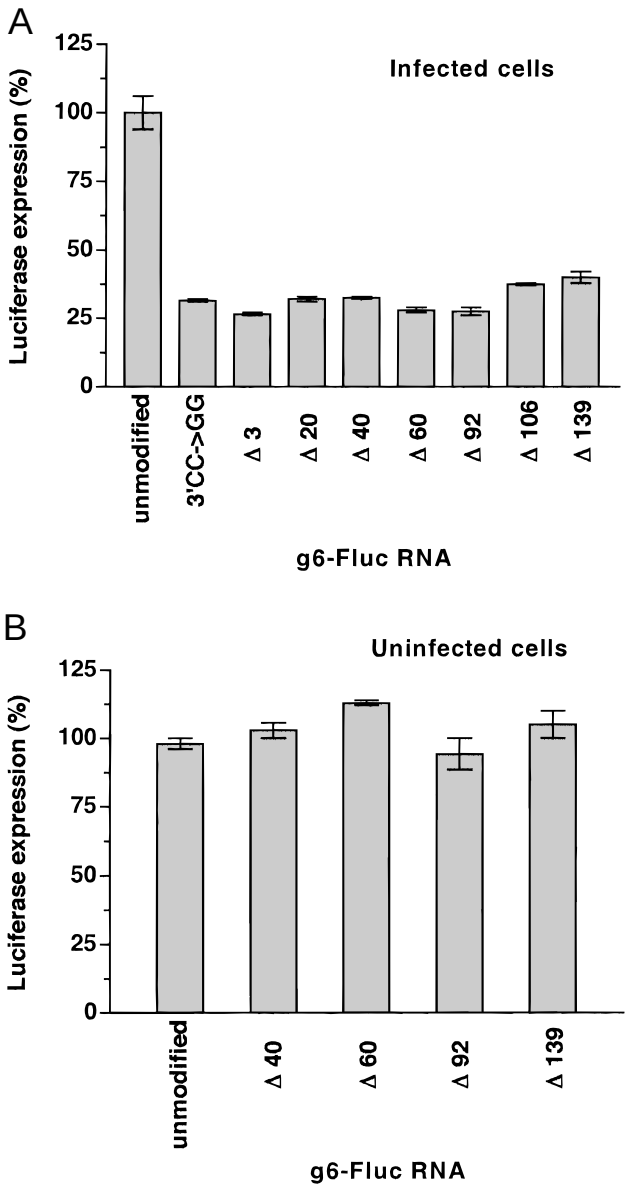

FIGURE 3. Effect of truncation of the 3′ UTR on protein expression in noninfected and infected cells. MA104 cells were rotavirus-infected (**A**) or mock-infected (**B**) and, at 1 h postinfection, were cotransfected with g6-Fluc RNA and nv-Rluc RNA or with a 3′-deletion mutant of g6-Fluc RNA and nv-Rluc RNA. The deletion mutants lacked from the last 3 (Δ3) to the last 139 (Δ139) bases of the 3′ end of the g6-Fluc RNA. The entire gene 6 3′ UTR is absent in the Δ139 RNA. Rotavirus-infected cells were also transfected with the mutant RNA, 3′CC → GG, that differed only from g6-Fluc RNA in that its last 2 nt, CC, were replaced with GG. At 9 h postinfection, the levels of firefly and *Renilla* luciferases per milligram of cell lysate were determined, and the expression of firefly luciferase was normalized to the expression of *Renilla* luciferase. To ease comparison of the values, the expression of firefly luciferase for the g6-Fluc RNA was set at 100%. The range reflects the values obtained in duplicate assays.

than in rotavirus-infected cells (Fig. 2A), production of the enzyme in mock-infected cells is measurable. As a consequence, we tested whether deletion of sequences from the 3′ end of g6-Fluc RNA reduced luciferase expression in mock-infected cells in a manner similar to that observed in infected cells. The results showed that deletions made at the 3′ end of the RNA, including one that removed the entire 3′ UTR (Δ139), had no impact on the production of firefly luciferase in mock-infected cells (Fig. 3B). Together, these findings indicated that the 3′ TE of g6-Fluc RNA operated in infected cells but not in uninfected cells.

3′-consensus sequence of viral mRNAs contains the 3′ TE

The sequence formed by the last seven bases of the g6-Fluc RNA is conserved among rotavirus mRNAs. Based on the results presented above, showing that the 3′ end of the g6 3′ UTR is important for efficient translation, we tested whether the 3′ TE was located in the 3′-conserved sequence. This was accomplished by removing the last 68 bases from the end of the g6-Fluc RNA (g6-FlucΔ68) and then evaluating the effect of adding sequences from the 3′ end of the g6 3′ UTR to the RNA on the expression of firefly luciferase in infected cells (Fig. 4A). Consistent with the data shown in Figure 3A, the absence of the last 68 bases of the 3′ UTR caused a three- to fourfold reduction in the expression of luciferase (Fig. 4B). When either the dinucleotide, AC, or the trinucleotide, ACC, which represents the last three bases of the gene 6 3′ UTR, were added to g6-FlucΔ68 RNA, expression of luciferase remained low. However, when the last four bases (GACC) of the gene 6 3′ UTR were added to g6-FlucΔ68 RNA, the expression of firefly luciferase in infected cells increased approximately threefold, reaching a level that was 75–80% of that of infected cells transfected with full-length g6-Fluc RNA (Fig. 4B). Hence, the sequence, GACC, which is found at the 3′ end of nearly all rotavirus mRNAs, contains sufficient information that allows it to operate as the 3′ TE.

Effect of 5′ UTR on gene expression

The 5′ UTR of the gene 6 mRNA is 23 bases in length and begins with a sequence common to all the viral mRNAs: GGC$(U/A)_7$. To determine the importance of the 5′ UTR on gene expression, the first 19 bases of the 5′ UTR of g6-Fluc RNA were replaced with the first 19 bases of the 5′ UTR of rhesus β-globin mRNA (glo/g6-Fluc RNA) (Martin et al., 1981) or with a random nonspecific sequence of 19 bases (syn/g6-Fluc RNA) (Fig. 1). Transfection of capped glo/g6-Fluc RNA and capped syn/g6-Flu RNA into infected cells resulted in the expression of luciferase at levels that were 60 and 80%, respectively, of the level of luciferase expressed in infected cells transfected with capped g6-Fluc RNA (Fig. 5). Thus, the presence of the viral 5′ UTR on the g6-Fluc RNA affected expression of luciferase by less

RNA	3′-Terminal Sequence
g6-Fluc	UAGAACUGUAUGAUGUGACC
g6-Fluc Δ68+15	UGUAACUGUAUGAUGUGACC
g6-Fluc Δ68+11	UGUAA AUGAUGUGACC
g6-Fluc Δ68+7	UGUAA UGUGACC
g6-Fluc Δ68+5	UGUAA UGACC
g6-Fluc Δ68+4	UGUAA GACC
g6-Fluc Δ68+3	UGUAA ACC
g6-Fluc Δ68+AC	UGUAA AC
g6-Fluc Δ68	UGUAA

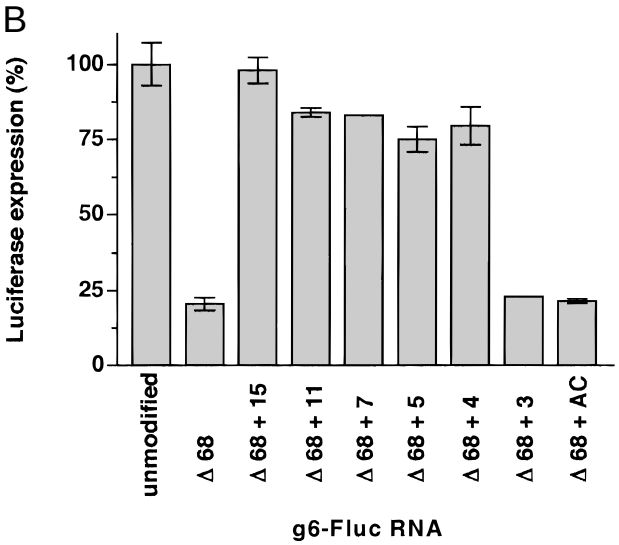

FIGURE 4. Importance of the 3′-terminal conserved sequence, GACC, on protein expression. **A**: The mutant g6-FlucΔ68 RNA lacks the 3′-terminal 68 bases of the g6-Fluc RNA. Derivatives of the mutant were made that contained the last 3 (g6-FlucΔ68+3) to 15 (g6-FlucΔ68+15) bases of the gene 6 3′ UTR added to the 3′ end of the g6-FlucΔ68 RNA. The g6-FlucΔ68+AC RNA was produced by adding the dinucleotide AC onto g6-FlucΔ68 RNA. **B**: Rotavirus-infected cells were cotransfected with the one of the gene 6 chimeric RNAs and with nv-Fluc RNA. At 9 h postinfection, the levels of firefly and *Renilla* luciferases per milligram of cell lysate were determined, and the expression of firefly luciferase was normalized to the expression of *Renilla* luciferase. The range reflects the values obtained in duplicate assays.

than twofold and was less of a factor in its expression than the 3′ TE (Fig. 4).

Rotavirus mRNAs that are translated into protein and are replicated to dsRNA contain 5′-terminal caps. To assess the importance of caps on protein expression, rotavirus-infected cells were cotransfected at 1 h postinfection either with capped g6-Fluc RNA and capped nv-Rluc RNA or with uncapped g6-Fluc RNA and capped nv-Rluc RNA, and the production of firefly and *Renilla* luciferase was measured at 9 h postinfection. The expression of firefly luciferase, normalized to the expression of *Renilla* luciferase, was 400-fold greater in cells transfected with capped than with uncapped g6-Fluc RNA (Fig. 6A). Because caps can affect the stability of RNAs, the possibility was examined that firefly luciferase was expressed at higher levels in the transfected cells, not because the capped RNA was translated more efficiently than the uncapped RNA, but because the capped RNA was more stable than the uncapped RNA. To compare the stability of the RNAs, rotavirus-infected cells were transfected with equal amounts of ^{32}P-labeled capped or uncapped g6-Fluc RNA. At 2, 5, and 8 h postinfection, RNA was recovered from the cells by phenol-chloroform extraction and was resolved by electrophoresis on a polyacrylamide-urea gel. The intensity of the bands of ^{32}P-labeled full-length capped and uncapped gene 6 Fluc RNAs detected on the gel were quantified with a PhosphorImager, and the values were plotted as a function of time. As shown in Figure 6B, no significant difference was detected in the recovery of full-length capped and uncapped g6-Fluc RNAs, indicating that RNA stability was not a factor in the difference in the expression of firefly luciferase by these RNAs. Considered together, these results indicate that

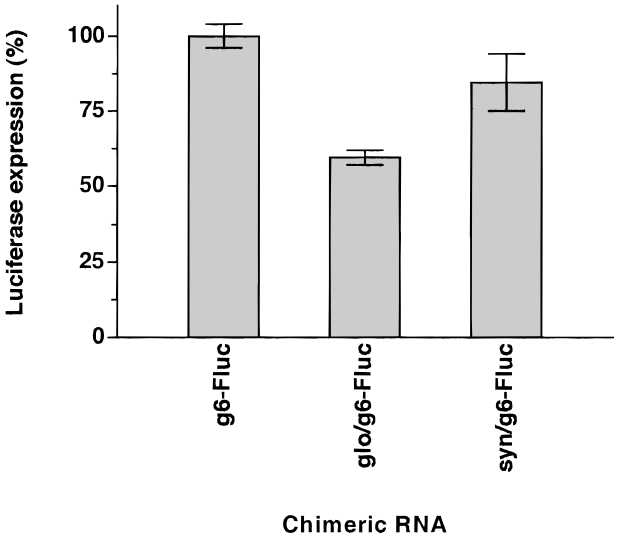

FIGURE 5. Effect of the gene 6 5′ UTR on gene expression. The RNAs, glo/g6-Fluc and syn/g6-Fluc, were made by replacing the 5′ UTR of g6-Fluc RNA with either the 5′ UTR of β-globin mRNA or with a random sequence, respectively (see Fig. 1). The UTRs of all the gene 6 chimeras were 23 bases. The gene 6 chimeric RNAs were cotransfected with nv-Rluc into rotavirus-infected cells and, at 9 h postinfection, the levels of firefly and *Renilla* luciferases per milligram of cell lysate were determined. The expression of firefly luciferase was normalized to the expression of *Renilla* luciferase. The range reflects the values obtained in duplicate assays.

efficient expression of firefly luciferase in the transfected cells was cap dependent.

Relationship between the 5′ UTR and 3′ TE

To further explore the question of whether the function of the 3′ TE required the 5′ UTR to be viral specific, deletions of 68 and 106 bases were made to the g6-Fluc and glo/g6-Fluc RNAs, resulting in the removal of the 3′ TE (Fig. 7A). These deletion-mutant RNAs were further modified by adding back the last 21 bases of the g6 3′ UTR, and therefore the 3′ TE, to their 3′ ends. The RNAs were cotransfected with nv-Rluc RNA into rotavirus-infected cells, and the expression of firefly and *Renilla* luciferases was measured at 9 h postinfection. For both the g6-Fluc and glo/g6-Fluc RNAs, deletion of the 3′-terminal 68 and 106 bases reduced the expression of firefly luciferase by a similar extent (three- to fivefold) (Fig. 7B,C). And when the last 21 bases of the gene 6 3′ UTR were added back to both the g6-Fluc and glo/g6-Fluc RNAs containing 3′ deletions (Δ68 and Δ106), the expression of luciferase was restored to levels at or near that obtained with the full-length g6-Fluc and glo/g6-Fluc RNA (Fig. 7B,C). Thus, the 3′ TE does not require viral-specific sequences at the 5′ end of the mRNA to promote protein expression. This result also rules out the possibility that the 3′ TE enhances expression by directly interacting through base pairing with the 5′ UTRs of viral mRNAs.

Effect of 3′-deletion mutagenesis on RNA stability

To address the possibility that g6-Fluc RNA and mutants of it directed the expression of different levels of firefly luciferase in infected cells because the RNAs differed in stability, capped ^{32}P-labeled g6-Fluc, g6-FlucΔ68, and g6-FlucΔ139 RNAs were prepared. The radiolabeled transcripts were transfected into rotavirus-infected cells and, at 3, 6, and 9 h postinfection, RNAs were recovered from the cells and were resolved by electrophoresis on a polyacrylamide-urea gel. The intensity of bands on the gel corresponding to the full-length ^{32}P-labeled RNAs were determined with a PhosphorImager and were plotted as a function of time. Based on the slopes of the lines on the plot, the stability of the full-length g6-Fluc RNA and mutant g6-Fluc RNAs lacking the 3′ TE were determined to be similar in the transfected cells (Fig. 8). Hence, the mechanism by which the 3′ TE operates to enhance luciferase expression is not related to its effect on RNA stability.

Chimeric RNAs are not templates for replication in vivo

Previous studies with a cell-free replication system have shown that the conserved seven-base sequence at the 3′ end of rotavirus RNAs contains essential and sufficient information to promote the synthesis of minus-strand RNA in vitro (Patton et al., 1996). Therefore, we examined the possibility that g6-Fluc RNA underwent replication when transfected into infected cells and, as a consequence, the expression of firefly luciferase was amplified because additional copies of the g6-Fluc RNA were produced in vivo. By electrophoretic analysis, no g6-Fluc dsRNA was detected in RNA recovered from infected cell lysates or from progeny virions (data not shown). Additionally, Northern blot analysis with ^{32}P-labeled plus-sense g6-Fluc RNA probes of the RNA recovered from infected cell lysates indicated that the g6-Fluc RNA was not replicated after transfection into rotavirus-infected cells (data not shown).

DISCUSSION

Communication between the 5′-cap structure and 3′ poly(A) tail of eukaryotic mRNAs enhances translation and is mediated by the interaction of proteins that bind to the 5′ cap (eIF-4G) and to the poly(A) tail (PABP) (Gallie, 1998). Although the viral mRNAs made by rotaviruses and other members of the *Reoviridae* contain caps, they lack poly(A) tails and therefore must use a different mechanism to achieve efficient translation. In this study, we have used a luciferase-encoding analog of the rotavirus gene 6 mRNA to identify sequences within viral UTRs that enhance translation. By mutagenesis, we showed that the 3′-terminal sequence,

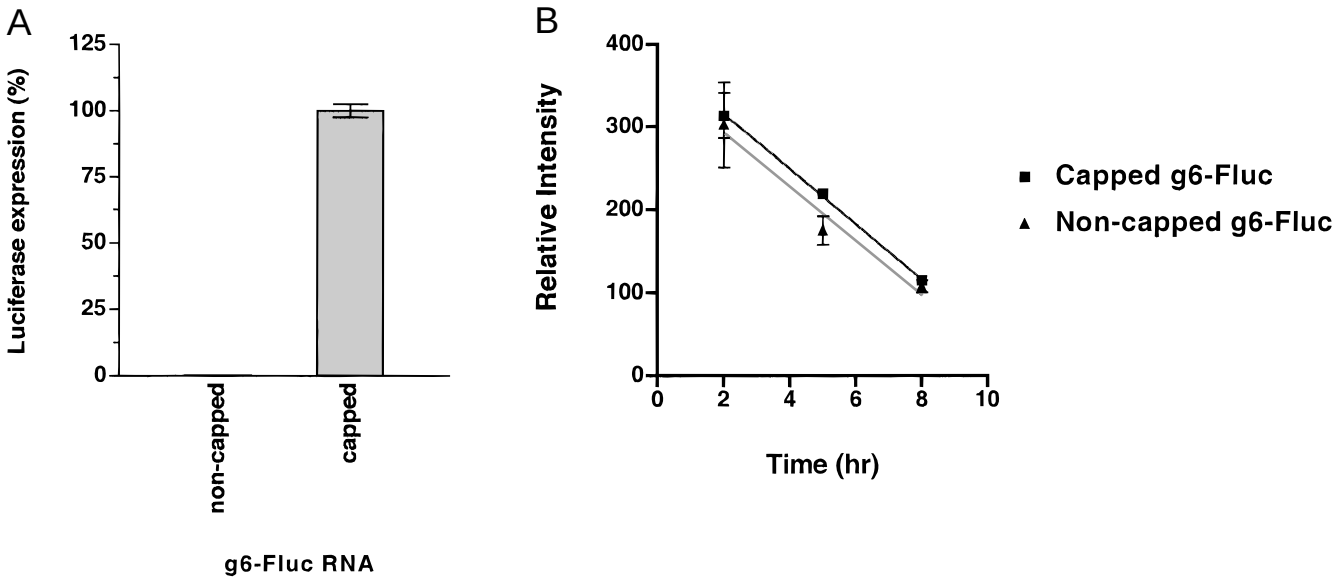

FIGURE 6. Effect of the 5′ cap on gene expression and RNA stability. **A**: Capped g6-Fluc RNA and capped nv-Rluc RNA or uncapped g6-Fluc RNA and capped nv-Rluc RNA were transfected into cells, and, at 9 h postinfection, the levels of firefly and *Renilla* luciferase expression were determined. The expression of firefly luciferase were normalized to the expression of *Renilla* luciferase. The range reflects the values obtained in duplicate assays. **B**: ^{32}P-labeled capped and uncapped g6-Fluc RNAs were transfected in duplicate into rotavirus-infected cells. At 2, 5, and 8 h postinfection, the RNAs were recovered from the cells and resolved by electrophoresis. The bands of ^{32}P-labeled full-length RNA were quantified with a PhosphorImager, and the values were plotted as a function of time. The range reflects the values obtained in duplicate assays.

GACC, of the gene 6 3′ UTR enhances translation, but only in rotavirus-infected cells, and thus represents a viral-specific 3′-TE element. The activity of the 3′ TE functioned independently of the sequence of the 5′ UTR, but efficient translation of the mRNA was cap dependent, even if the enhancer was present on the mRNA. Because the 3′-terminal sequence of nearly all group A rotavirus mRNAs reported to date is GACC, it is likely that the 3′ TE represents a common element used to up-regulate the expression of viral proteins in the infected cell. Although 3′-TE elements have been identified in other viral and cellular mRNAs, the 4-nt sequence of the rotavirus 3′ TE is remarkable, representing by far the shortest of any of the sequence enhancers known to stimulate translation.

Computer modeling of the secondary structure of rotavirus mRNAs (Mathews et al., 1999; Zuker et al., 1999) has indicated that the 3′-terminal sequence GACC is neither base paired with other portions of the RNA nor is part of any stem-loop or other higher order structure (Chen & Patton, 1998; data not shown). So far, the only feature of the rotavirus 3′ TE that appears to be important for its function is its primary sequence. In contrast to the rotavirus 3′ TE, the enhancers of the 3′ UTRs of histone, AMV, and TMV mRNAs appear to be associated with longer sequences that have higher-order structures and that reside further upstream from the 3′ end (Leathers et al., 1993; Pandey et al., 1994; Williams & Marzluff, 1995; Hann et al., 1997).

In uninfected cells, the expression of luciferase from the g6-Fluc RNA was inefficient, and in infected cells, the expression of luciferase from the g6-Fluc RNA correlated with the expression of viral proteins (Fig. 2A). These results suggest that one or more of the viral proteins plays a role in the activity of the 3′ TE. The identity of the protein(s) remains to be determined, but most likely includes one or more of the viral structural (VP1, VP2, and VP3) or nonstructural (NSP1, NSP2, and NSP3) RNA-binding proteins (Patton, 1995; Patton & Chen, 1999). Given their specific affinity for the 3′ ends of rotavirus, the two most likely candidates are VP1 and NSP3. Indeed, previous studies by Poncet et al. (1994) have shown that NSP3 specifically recognizes the 3′-terminal sequence GACC (Poncet et al., 1994), the same sequence shown in this study to operate as a 3′ TE. Additionally, NSP3 has been shown by the two-hybrid system and by pull-down assays to interact with eIF-4GI (Piron et al., 1998, 1999); therefore, NSP3 would appear to be a functional homolog of PABP in that both proteins have affinity for the 3′ end of mRNA and for a cap-associated initiation factor. Based on its RNA- and protein-binding activities, it may be speculated that NSP3 participates in the circularization of rotavirus mRNAs in polysomes, and thereby increases the efficiency of translation. Our results are the first to provide evidence that the NSP3-binding site of the viral mRNA, that is, the 3′ TE, operates to increase the efficiency of translation in vivo. We found no evi-

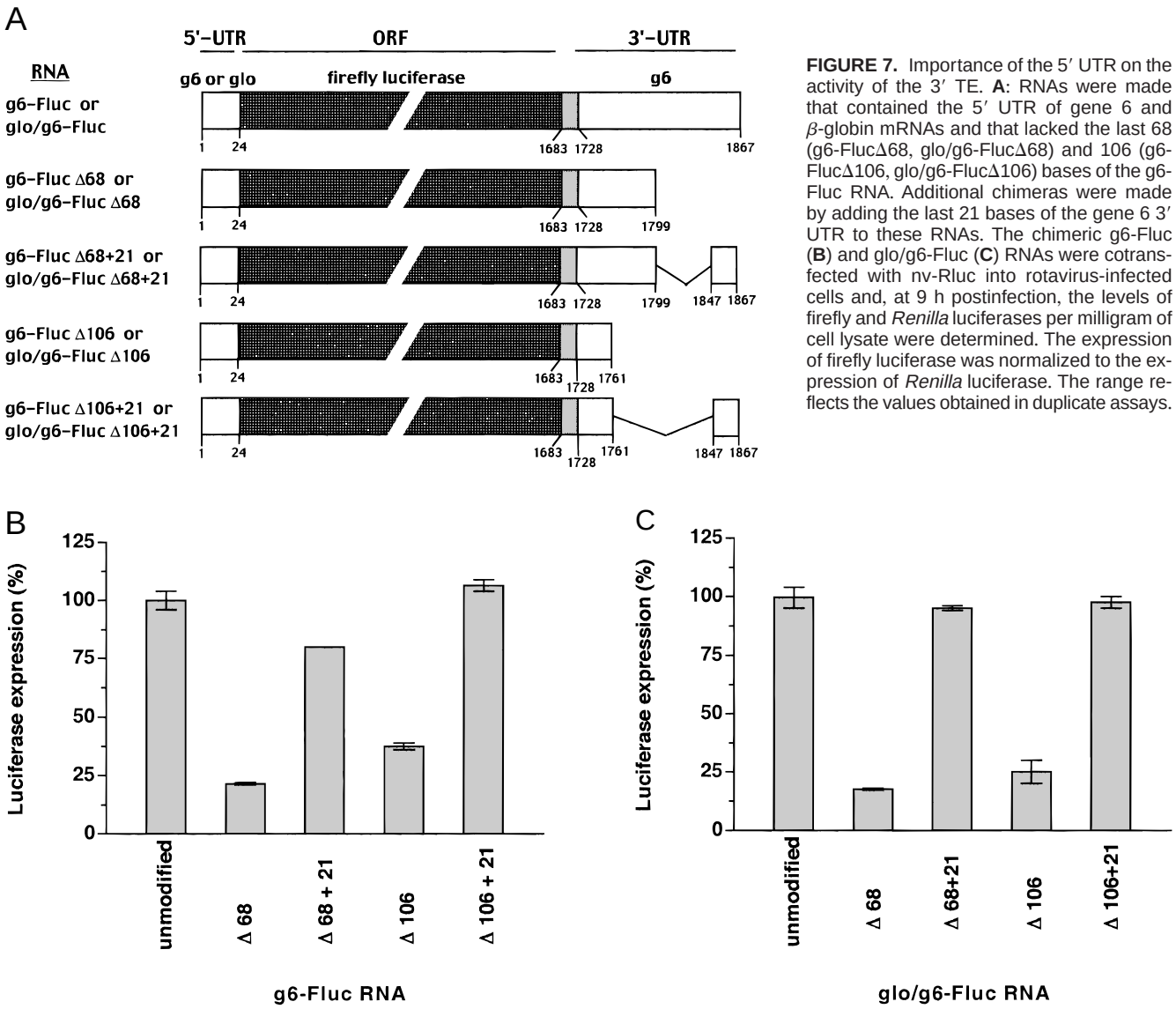

FIGURE 7. Importance of the 5′ UTR on the activity of the 3′ TE. **A**: RNAs were made that contained the 5′ UTR of gene 6 and β-globin mRNAs and that lacked the last 68 (g6-FlucΔ68, glo/g6-FlucΔ68) and 106 (g6-FlucΔ106, glo/g6-FlucΔ106) bases of the g6-Fluc RNA. Additional chimeras were made by adding the last 21 bases of the gene 6 3′ UTR to these RNAs. The chimeric g6-Fluc (**B**) and glo/g6-Fluc (**C**) RNAs were cotransfected with nv-Rluc into rotavirus-infected cells and, at 9 h postinfection, the levels of firefly and *Renilla* luciferases per milligram of cell lysate were determined. The expression of firefly luciferase was normalized to the expression of *Renilla* luciferase. The range reflects the values obtained in duplicate assays.

dence that the 3′ TE increased the efficiency of translation by increasing the stability of nonpolyadenylated viral mRNAs.

Because the interaction of NSP3 with eIF-4GI disrupts the interaction of eIF-4F and PABP, Piron et al. (1998) proposed that the formation of circular polysomes with cellular polyadenylated mRNAs in infected cells could be inhibited, leading to a decrease of host cell protein synthesis. Our experiments do not address whether there is in fact a change in the efficiency of translation of polyadenylated mRNAs in infected cells. Although our studies showed that the expression of firefly luciferase from the g6-Fluc RNA was 75 times greater in infected cells than uninfected cells at 11 h postinfection, we also observed that the expression of *Renilla* luciferase from a nonpolyadenylated chimeric RNA containing no viral sequences, that is, nv-Rluc, was 15 times greater in infected cells than in uninfected cells (Fig. 2A). This suggests that rotavirus infection may lead to a general up-regulation in the translation of nonpolyadenylated mRNAs in the cell via a process that does not involve the 3′ TE. Thus, both specific and nonspecific mechanisms may function to promote the translation of viral mRNAs in infected cells.

The development of rotavirus cell-free replication systems has provided considerable information as to the location of *cis*-acting signals in rotavirus mRNA that promote the synthesis of minus-strand RNA, resulting in the formation of dsRNA (Chen et al., 1994). The results of such studies have indicated that *cis*-acting replication signals are located in both the 5′ and 3′ UTRs of viral mRNAs and that the 3′-terminal conserved sequence of the mRNAs is essential for minus-strand synthesis (Patton et al., 1996; Wentz et al., 1996). Not only does NSP3 have affinity for the 3′ end of the mRNA, but based on gel mobility shift assays, the viral

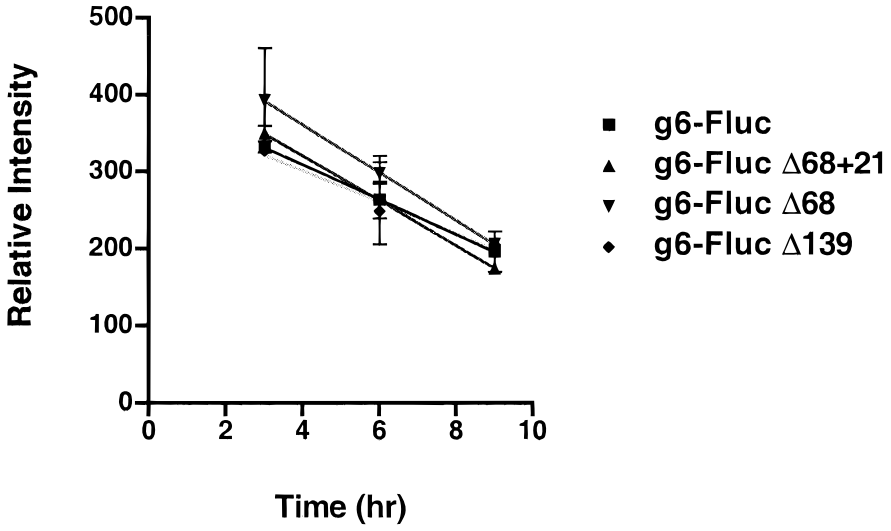

FIGURE 8. Effect of deletion mutagenesis on RNA stability. ^{32}P-labeled g6-Fluc, g6-FlucΔ68, and g6-FlucΔ139 RNAs were transfected into rotavirus-infected cells. At 3, 6, and 9 h postinfection, RNAs were recovered from the cells and resolved by electrophoresis. The bands of ^{32}P-labeled full-length RNA were quantified with a PhosphorImager, and the values were plotted as a function of time. The range reflects the values obtained in duplicate assays.

RNA polymerase, VP1, does as well (Patton, 1996). Because rotavirus mRNAs serve dual functions in the infected cell, it may be reasonable to propose that whether the mRNA is replicated or translated is determined by competition between VP1 and NSP3 for the 3′ end. However, that may not be the case, as VP1 accumulates in cytoplasmic inclusions, termed viroplasms, that form in infected cells and that are the sites of RNA replication and capsid assembly, whereas NSP3 accumulates on the cytoskeleton (Mattion et al., 1992), the presumed site of mRNA translation. If replication and translation occur in different places in the cell, then in fact VP1 and NSP3 may never compete for the same molecule of mRNA.

In an exhaustive effort to generate a reverse genetics system for the rotaviruses by transfection of chimeric reporter RNAs and wild-type viral mRNAs into infected cells, we have found that although the RNAs can be translated, they are neither replicated into dsRNAs nor incorporated into progeny virions. Thus, transfected RNAs can move to the site of translation in the infected cell, but apparently cannot move to the site of replication. These findings raise the possibility that trafficking of viral mRNAs to the site of replication proceeds by a pathway separate from that which is used to move viral mRNAs to the site of translation.

In a previous study, Gorziglia and Collins (1992) produced an analog RNA that contained the 5′ and 3′ UTRs of the gene 6 mRNA and an ORF for chloramphenicol acetyltransferase (CAT). Like the results obtained with the g6-Fluc RNA, these investigators found that CAT expression was higher in infected than uninfected cells. They interpreted their results to indicate that the enhancement of CAT expressions stemmed from the g6-CAT analog RNA undergoing replication by the viral polymerase, resulting in amplification of the RNA. However, no direct evidence was provided in their study that the g6-CAT analog RNA had been used as a template for minus-strand synthesis, to form dsRNA. Based on our studies, we believe that the enhanced expression of CAT that they observed was because of up-regulation of translation by the 3′ TE of the g6-CAT RNA in the infected cell. Despite extensive efforts, we have obtained no evidence that the plus-sense g6-Fluc RNA was replicated, producing either minus-strand RNA or to dsRNA in infected cells. Therefore, we conclude that the amplification of firefly luciferase in infected cells is the result of the enhancement of translation and not the amplification of the RNA by replication.

In summary, the sequence GACC is common to the 3′ ends of nearly all rotavirus mRNAs, and probably functions as a general enhancer to increase the expression of all the viral proteins. However, because the quantitative demand for each of the viral proteins in the rotavirus-replication cycle varies considerably, we expect that other elements will be identified in the viral mRNAs that will regulate translation efficiency. Whether these elements will operate independently of the 3′ TE or in concert with the 3′ TE by stimulating or impeding the interaction of NSP3 with eIF-4GI will be of interest.

MATERIALS AND METHODS

Cells and viruses

Fetal rhesus monkey kidney (MA104) cells were maintained in M199 medium supplemented with 7% fetal bovine serum. Stocks of simian rotavirus SA11 and rhesus rotavirus RRV were prepared as described earlier and were titered by plaque assay (Helmberger-Jones & Patton, 1986).

Construction of plasmids

The plasmid SP72g6 contains a full-length cDNA of the gene encoding VP6 (segment 6) of SA11 rotavirus and was constructed as described by Mansell et al. (1994). To generate

the T7 transcription vector, pVec2.0g6 (*Nco*I), the gene 6 cDNA in SP72g6.1 was amplified with the High Fidelity PCR System (Roche Molecular Biochemicals) and the primer pair, s6P and s6M (Table 1). T4 DNA ligase was then used to blunt-end ligate the amplified cDNA into the *Stu*I and *Sma*I restriction sites of the vector, pV2.0 (Sambrook et al., 1992). pV2.0 is a derivative of pMJ5 (Allison et al., 1988) and was kindly provided to us by Dr. Andrew Ball, University of Alabama at Birmingham. The appropriate sequence was included in s6P to introduce a cleavage site for *Nco*I immediately upstream of the ORF in the gene 6 cDNA. As a result, the gene 6 cDNA in pVec2.0g6 contains an A → C mutation at residue 22.

The vector, pVec2.0g6-Fluc, was constructed by replacing the VP6 ORF in pVec2.0g6 (*Nco*I) with the ORF for firefly luciferase (Fig. 1). A DNA fragment containing the ORF was generated by digesting the luciferase expression vector, pGL3-Basic (Promega), with *Xba*I, treating the linearized plasmid with T4 DNA polymerase to blunt-end the terminal overhangs, and then digesting the plasmid with *Nco*I. After gel purification, the fragment was ligated into pVec2.0g6 (*Nco*I), which had been digested with *Mlu*I, treated with T4 DNA polymerase and then digested with *Nco*I. Sequences of the vectors were examined with an ABI automated DNA sequencer.

The T7 transcription vector, pRL-null (Promega), contains the ORF for *Renilla* luciferase and, following linearization with *Bam*H1, was transcribed with T7 RNA polymerase to produce nv-Rluc RNA.

PCR-directed mutagenesis of the 5′ and 3′ UTRs of the gene 6-Fluc RNAs

The T7-transcription template used to produce the gene 6 analog RNA, g6-Fluc, was synthesized by PCR amplification with the primers T7-5′ UTR and m6M (Table 1) and the plasmid pVec2.0g6-Fluc (Fig. 1). Except for the mutation A22 → C, the 5′ and 3′ UTRs of g6-Fluc RNA are the same as that of authentic SA11 gene 6 mRNA. To alter the 5′ and 3′ UTRs of the g6-luc RNA, PCR amplifications were performed with the various primers listed in Table 1 and with pVec2.0g6-Fluc. The amplification reactions were carried out with the High Fidelity PCR System (Roche Molecular Biochemicals), and the PCR products were purified with a QIAquick PCR purification kit (Qiagen). The positive-sense primers contained the promoter sequence for T7 RNA polymerase, thus allowing the amplification products to be directly transcribed with T7 RNA polymerase.

In vitro synthesis of RNAs

Capped and noncapped RNAs were synthesized from PCR products containing T7 promoters using the Ambion mMESSAGE and MEGAscript transcription systems, respectively. Transcripts were radiolabeled by adding 1 μCi of [α-^{32}P]UTP (800 Ci/mmol) to reaction mixtures. DNA templates were removed from transcription reaction mixtures by treating with DNase I. The RNAs were then recovered by phenol-chloroform extraction and isopropanol precipitation. The RNA samples were passed through G-50 spin columns (5 Prime → 3 Prime, Inc.) to remove unincorporated nucleotides. The concentration of RNAs was estimated with a spectrophotometer. Subsequently, the ^{32}P-labeled transcripts were resolved by electrophoresis on 5% polyacrylamide gels containing 7 M urea (Chen et al., 1994) and quantified with a PhosphorImager. The results were then used to adjust the concentration of the RNAs to assure that equal amounts of transcript were used in transfection experiments.

TABLE 1. Oligonucleotide primers used in preparation of T7 transcripts.

Primer	Sequence	RNA
m6P	cttttaaacgaagtcttcaccatggatgtcct	g6-Fluc
m6M	ggtcacatcctctcactataccatctgagtg	g6-Fluc
T7-5′ UTR	cccaggtaccctaatacgactcactataggcttttaaacgaagtcttcaccatg	g6-Fluc
T7-β-globin	cccaggtaccctaatacgactcactatagacacttgcttctgacacacaccatggaagacgccaaaaacataaag	glo/g6-Fluc
T7-synthetic	cccaggtaccctaatacgactcactatagatctaatattgttcttctcaccatggaagacgccaaaaacata	syn/g6-Fluc
3′ CC → GG	cctcacatcctctcactataccatctg	g6-Fluc-3′CC → GG
Del 3	cacatcctctcactataccat	g6-Fluc Δ3
Del 20	ccatcagagtgattactctgtgta	Fluc Δ20
Del 40	tgtagcgaatgcgtatacgcatcc	g6-Fluc Δ40
Del 60	atccttacttacagagttcaaaca	g6-Fluc Δ60
Del 68	ttacagagttcaaacagcttg	g6- and glo/g6-Fluc Δ68
Del 68 + AC	gtttacagagttcaaacagcttgatatag	g6-Fluc Δ68 + AC
Del 68 + 3	ggtttacagagttcaaacagcttg	g6-Fluc Δ68 + 3
Del 68 + 4	ggtcttacagagttcaaacagcttg	g6-Fluc Δ68 + 4
Del 68 + 5	ggtcattacagagttcaaacagcttg	g6-Fluc Δ68 + 5
Del 68 + 7	ggtcacatttacagagttcaaacagcttg	g6-Fluc Δ68 + 7
Del 68 + 11	ggtcacatcctttacagagttcaaacagcttg	g6-Fluc Δ68 + 11
Del 68 + 15	ggtcacatcctctcattacagagttcaaacagcttg	g6-Fluc Δ68 + 15
Del 68 + 21	ggtcacatcctctcactatacttacagagttcaaacagcttg	g6- and glo/g6-Fluc Δ68 + 21
Del 92	tagctacatactcaccaa	g6-Fluc Δ92
Del 106	ccaaagttggataccaagttgttagc	g6- and glo/g6-Fluc Δ106
Del 106 + 21	ggtcacatcctctcactatacccaaagttggataccaagttgttagc	g6- and glo/g6-Fluc Δ106 + 21
Del 139	tcatttaatgagcatgcttctaatgga	g6-Fluc

Infection and transfection of MA104 cells

After reaching 70–80% confluency in 12-well plates, MA104 monolayers were mock-infected or infected with trypsin-activated SA11 rotavirus for 1 h at a multiplicity of infection of 5. After washing with M199 medium, a liposome-RNA transfection mixture prepared by combining a total of 1 to 1.5 μg of RNA, 3 μL of Plus reagent, 3 μL lipofectAMINE (Life Technologies), and 0.35 mL of M199 medium was placed in each well. After incubation for 2 h at 37 °C, the transfection mixture was replaced with 0.7 mL of M199 containing 10% fetal bovine serum, and the incubation continued.

Luciferase assays

To measure the expression of firefly and *Renilla* luciferases, 12-well plates containing transfected MA104 cells were chilled in an ice-water bath, and the cells were harvested by scraping into cold phosphate-buffered saline (PBS). Cells from each well were pelleted by centrifugation at 780 $\times$ *g* for 3 min at 4 °C and resuspended in 30 μL of 1$\times$ Passive Lysis Buffer (PLB) provided in the Dual Luciferase Kit (Promega). The activity of both firefly and *Renilla* luciferases was assayed according to the protocols of the supplier using a Turner TD-20E luminometer. The luminometer was programmed to give a 10-s measurement period after a 5-s premeasurement period.

RNA stability assays

MA104 cells were infected with SA11 rotavirus and transfected with equal amounts of ^{32}P-labeled RNA as described above. At the indicated times, the cells were washed twice with PBS, then lysed in a 14:100 mixture of PBS and Trizol. The lysates were extracted sequentially with chloroform, phenol, and chloroform, and RNA was recovered from the extracts by precipitation with isopropanol. The RNA pellets were resuspended in water and analyzed by electrophoresis on 5% polyacrylamide gels containing 7 M urea (Patton et al., 1996). The intensity of bands of radiolabeled RNAs identified on gels was determined using a Molecular Dynamics PhosphorImager.

Viral protein synthesis in infected cells

Monolayers of MA104 cells in 6-well plates were mock-infected or infected with 20 plaque-forming units per cell of SA11 rotavirus and, beginning at 1 h postinfection, maintained continuously in 90% Met- and Cys-free minimal essential medium (MEM) containing 40 μCi per mL of ^{35}S-methionine (^{35}S-Express, NEN, 1,175 mCi/mmol). After rinsing the monolayers twice with cold PBS, the cells were resuspended in hypotonic buffer (3 mM Tris-HCl, pH 8.5, 0.5 mM $MgCl_2$, 3 mM NaCl) containing 0.5% Triton X-100 and were incubated for 3 min on ice. Nuclei and large debris were moved from the lysates by centrifugation at 1,000 $\times$ *g* for 1 min. ^{35}S-labeled proteins were detected in the lysates by electrophoresis on 12% polyacrylamide gels containing SDS (Patton et al., 1999) and by autoradiography.

ACKNOWLEDGMENTS

We thank Zenobia Taraporewala for valuable input into the project and Melinda Jones for technical assistance.

Received October 22, 1999; returned for revision December 9, 1999; revised manuscript received March 21, 2000

REFERENCES

Allison RF, Janda M, Ahlquist P. 1988. Infectious in vitro transcripts from cowpea chlorotic mottle virus cDNA clones and exchange of individual RNA components with brome mosaic virus. *J Virol 62*:3581–3588.

Chen D, Patton JT. 1998. Rotavirus RNA replication requires a single-stranded 3′ end for efficient minus-strand synthesis. *J Virol 72*:7387–7396.

Chen DY, Zeng CQ-Y, Wentz MJ, Gorziglia M, Estes MK, Ramig RF. 1994. Template-dependent, in vitro replication of rotavirus RNA. *J Virol 68*:7030–7039.

Desselberger U, McCrae MA. 1994. The rotavirus genome. *Curr Top Microbiol Immunol 185*:31–66.

Estes MK. 1996. Rotaviruses and their replication. In: Fields BN, Knipe DM, Howley PM, eds. *Fundamental virology.* 3rd ed. Philadelphia, Pennsylvania: Lippincott-Raven Publishers. pp 731–761.

Gallie DR. 1991. The cap and poly(A) tail function synergistically to regulate mRNA translational efficiency. *Genes & Dev 5*:2108–2116.

Gallie DR. 1998. A tale of two termini: A functional interaction between the termini of an mRNA is a prerequisite for efficient translation initiation. *Gene 216*:1–11.

Gallie DR, Walbot V. 1990. RNA pseudoknot domain of tobacco mosaic virus can functionally substitute for a poly(A) tail in plant and animals. *Genes & Dev 4*:1149–1157.

Gorziglia M, Collins P. 1992. Intracellular amplification and expression of a synthetic analog of rotavirus genomic RNA bearing a foreign marker gene: Mapping *cis*-acting nucleotides in the 3′-noncoding region. *Proc Natl Acad Sci USA 89*:5784–5788.

Hann L, Webb AC, Cai J-M, Gehrke L. 1997. Identification of a competitive translation determinant in the 3′ untranslated region of alfalfa mosaic virus coat protein mRNA. *Mol Cell Biol 17*:2005–2013.

Helmberger-Jones M, Patton JT. 1986. Characterization of subviral particles in cells infected with simian rotavirus SA11. *Virology 155*:655–665.

Imai MK, Akatani K, Ikegami N, Furuichi Y. 1983. Capped and conserved terminal structures in human rotavirus double-stranded RNA segments. *J Virol 47*:125–136.

Imataka H, Gradi A, Sonenberg N. 1998. A newly identified N-terminal amino acid sequence of human eIF4G binds poly(A)-binding protein and functions in poly(A)-dependent translation. *EMBO J 17*:7480–7489.

Jacobson A. 1996. Poly(A) metabolism and translation: The closed-loop model. In: Hershey JWB, Mathews MB, Sonenberg N, eds. *Translation control.* Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 451–480.

Johnson MA, McCrae MA. 1989. Molecular biology of rotaviruses. VIII. Quantitative analysis of regulation of gene expression during virus replication. *J Virol 63*:2048–2055.

Le H, Tanguay RL, Balasta ML, Wei C-C, Browning KS, Metz AM, Goss DJ, Gallie DR. 1997. Translation initiation factors eIF-iso4G and eIF-4B interact with poly(A)-binding protein and increase its RNA binding activity. *J Biol Chem 272*:16247–16255.

Leathers V, Tanguay R, Kobayashi M, Gallie D. 1993. A phylogenetically conserved sequence within viral 3′ untranslated RNA pseudoknots regulates translation. *Mol Cell Biol 13*:5331–5347.

Mansell EA, Ramig RF, Patton JT. 1994. Temperature-sensitive lesions in the capsid proteins of the rotavirus mutants tsF and tsG that affect virion assembly. *Virology 204*:69–81.

Martin, SL, Zimmer EA, Davidson WS, Wilson AC, Kan YW. 1981. The untranslated regions of beta-globin mRNA evolve at a functional rate in higher primates. *Cell 25*:737–741.

Mathews DH, Sabina J, Zuker M, Turner DH. 1999. Expanded se-

quence dependence of thermodynamic parameters provides robust prediction of RNA secondary structure. *J Mol Biol 288*:911–940.
Mattion NM, Cohen J, Aponte C, Estes MK. 1992. Characterization of an oligomerization domain and RNA-binding properties on rotavirus nonstructural protein NS34 (NSP3). *Virology 190*:68–83.
McCrae MA, McCorquodale JG. 1983. Molecular biology of rotaviruses. V. Terminal structure of viral RNA species. *Virology 126*: 204–212.
Meulewaeter F, Van Montagu M, Cornelissen M. 1998. Features of the autonomous function of the translational enhancer domain of satellite tobacco necrosis virus. *RNA 4*:1347–1356.
Mitchell DB, Both GW. 1988. Simian rotavirus SA11 segment 11 contains overlapping reading frames. *Nucleic Acids Res 16*:6244.
Pandey NB, Williams AS, Sun J-H, Brown VD, Bond U, Marzluff WF. 1994. Point mutations in the stem-loop at the 3′ end of mouse histone mRNA reduce expression by reducing the efficiency of 3′ end formation. *Mol Cell Biol 14*:1709–1720.
Patton JT. 1995. Structure and function of the rotavirus RNA-binding proteins. *J Gen Virol 76*:2633–2644.
Patton JT. 1996. Rotavirus VP1 alone specifically binds to the 3′ end of viral mRNA but the interaction is not sufficient to initiate minus-strand synthesis. *J Virol 70*:7940–7947.
Patton JT, Chen D. 1999. RNA-binding and capping activities of proteins in rotavirus open cores. *J Virol 73*:1382–1391.
Patton JT, Taraporewala Z, Chizhikov V, Chen D. 1999. Virus replication. In: Gray J, Desselberger U, eds. *Rotaviruses: Methods and protocols*. Totowa, New Jersey: Humana Press. pp. 33–66.
Patton JT, Wentz M, Xiaobo J, Ramig RF. 1996. *Cis*-acting signals that promote genome replication in rotavirus mRNA. *J Virol 70*:3961–3971.
Piron M, Delaunay T, Grosclaude J, Poncet D. 1999. Identification of the RNA-binding, dimerization, and eIF4GI-binding domains of rotavirus nonstructural protein NSP3. *J Virol 73*:5411–5421.
Piron M, Vende P, Cohen J, Poncet D. 1998. Rotavirus RNA-binding protein NSP3 interacts with eIF4GI and evicts the poly(A) binding protein from eIF4F. *EMBO J 17*:5811–5821.
Poncet D, Aponte C, Cohen J. 1993. Rotavirus protein NSP3 (NS34) is bound to the 3′ end consensus sequence of viral mRNAs in infected cells. *J Virol 67*:3159–3165.
Poncet D, Laurent S, Cohen J. 1994. Four nucleotides are the minimal requirement for RNA recognition by rotavirus non-structural protein NSP3. *EMBO J 13*:4165–4173.
Preiss T, Hentze MW. 1998. Dual function of the messenger RNA cap structure in poly(A)-tail-promoted translation in yeast. *Nature 392*:516–520.
Sambrook J, Fritsch EF, Maniatis T. 1992. *Molecular cloning: A laboratory manual*. 2nd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.
Tarun Jr SZ, Sachs AB. 1996. Association of the yeast poly(A) tail binding protein with translation initiation factor eIF-4G. *EMBO J 15*:7168–7177.
Wang S, Guo L, Allen E, Miller WA. 1999. A potential mechanism for selective control of cap-independent translation by a viral RNA sequence in *cis* and in *trans*. *RNA 5*:728–738.
Wells SE, Hillner PE, Vale RD, Sachs AB. 1998. Circularization of mRNA by eukaryotic translation initiation factors. *Mol Cell 2*:135–140.
Wentz MJ, Patton JT, Ramig RF. 1996. The 3′-terminal consensus sequence of rotavirus mRNA is the minimal promoter of negative-strand RNA synthesis. *J Virol 70*:7833–7841.
Williams AS, Marzluff WF. 1995. The sequence of the stem and flanking sequences at the 3′ end of histone mRNA are critical determinants for the binding of the stem-loop binding protein. *Nucleic Acids Res 23*:654–662.
Zuker M, Mathews DH, Turner DH. 1999. Algorithms and thermodynamics for RNA secondary structure prediction: A practical guide. In: Barciszewski J, Clark BFC, eds. *RNA biochemistry and biotechnology*. Dordrecht, Netherlands: NATO ASI Series, Kluwer Academic Publishers. pp 11–43.