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Rotavirus Prevents the Expression of Host Responses by Blocking the Nucleocytoplasmic Transport of Polyadenylated mRNAs

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Rotaviruses are the most important agent of severe gastroenteritis in young children. Early in infection, these viruses take over the host translation machinery, causing a severe shutoff of cell protein synthesis while viral proteins are efficiently synthesized. In infected cells, there is an accumulation of the cytoplasmic poly(A)-binding protein in the nucleus, induced by the viral protein NSP3. Here we found that poly(A)-containing mRNAs also accumulate and become hyperadenylated in the nuclei of infected cells. Using reporter genes bearing the untranslated regions (UTRs) of cellular or viral genes, we found that the viral UTRs do not determine the efficiency of translation of mRNAs in rotavirus-infected cells. Furthermore, we showed that while a polyadenylated reporter mRNA directly delivered into the cytoplasm of infected cells was efficiently translated, the same reporter introduced as a plasmid that needs to be transcribed and exported to the cytoplasm was poorly translated. Altogether, these results suggest that nuclear retention of poly(A)-containing mRNAs is one of the main strategies of rotavirus to control cell translation and therefore the host antiviral and stress responses.

Due to their limited coding capacity, viruses are obligate intracellular parasites and depend on the cell translation machinery to synthesize their proteins. The replication of a virus requires that viral mRNAs compete successfully with cellular mRNAs for the host translation apparatus. Viruses have developed remarkable strategies to ensure the efficient translation of their mRNAs while simultaneously inhibiting cellular protein synthesis. While every step of the translation process is amenable to regulation, under most circumstances mRNA translation is regulated primarily at the level of initiation (1). Translation initiation is a complex process that begins with the recognition of the cap nucleotide structure (m⁷GpppN) at the 5' end of mRNAs by the cap binding protein eIF4E, which is part of the cap-binding complex eIF4F. This complex is composed of eIF4E, the ATP-dependent RNA helicase eIF4A, and the scaffolding protein eIF4G. eIF4G also interacts with the cytoplasmic poly(A)-binding protein (PABPC), which in turn binds to the 3' poly(A) tail of mRNAs. These interactions are thought to promote the circularization of the mRNA molecules to stimulate translation (2, 3). The binding of Met-tRNA to the 40S ribosomal subunit is mediated by a ternary complex composed of eIF2-GTP-Met-tRNA. Once the 40S ribosomal subunit is bound to the mRNA, it is thought to scan the mRNA in the 5'-to-3' direction (4). The AUG codon that is most proximal to the cap and flanked by optimal consensus sequences is usually selected for initiation (5). The release of eukaryotic translation initiation factors (eIFs) is assisted by eIF5, which facilitates the hydrolysis of GTP carried out by eIF2. The GDP on eIF2 is exchanged for GTP by eIF2B in a regulated manner that is essential for ensuing rounds of initiation (6). Different kinds of stress reduce global translation by triggering the phosphorylation of the α subunit of eIF2 (eIF2 α). This phosphorylation inhibits the exchange of GDP for GTP catalyzed by eIF2B, which is then sequestered in a complex with eIF2, preventing translation reinitiation. Even though PABPC is predominantly cytoplasmic, this protein has been detected in the nuclei of mammalian cells associated with nuclear pre-mRNPs (7–11). Thus, PABPC is regarded as a shut-

ting protein that participates in the maturation and nuclear export of mRNAs.

Rotaviruses are the most important cause of viral diarrhea in infants and young children, being responsible for an estimated incidence of 600,000 annual deaths worldwide (12) and placing a significant economic burden on the global health care system. These nonenveloped viruses are formed by three concentric layers of protein that enclose a genome composed of 11 segments of double-stranded RNA (dsRNA). The transcripts made by these viruses serve two functions in infected cells: as mRNAs to direct the synthesis of viral proteins and as templates for the synthesis of the minus-strand RNA to replicate the genome. Except for segment 11 mRNA, which is dicistronic, the mRNAs of rotavirus are monocistronic, coding for a total of six structural (VP1 to VP4, VP6, and VP7) and six nonstructural (NSP1 to NSP6) proteins (13).

Early in the infection process, rotaviruses take over the host translation machinery, causing a severe shutoff of cell protein synthesis. Rotavirus mRNAs are capped, but they are not polyadenylated; instead, they have a consensus sequence at their 3' end (GACC) that is conserved in all 11 viral genes (14) and which is bound by the amino-terminal domain of the viral nonstructural protein NSP3. This protein also binds, through its carboxy-terminal domain, to eIF4GI, in the same region used by PABPC but with higher affinity (15, 16); thus, it was proposed that during rotavirus infection, NSP3 evicts PABPC from eIF4GI, impairing the translation of cellular mRNAs while leading to the enhancement of translation of rotaviral mRNAs (15, 17, 18). Furthermore, it has been reported that in rotavirus-infected cells, PABPC accu-

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mulates in the cell nucleus, and this relocalization depends on NSP3 through a still-unknown mechanism (19, 20). We have found that while NSP3 indeed blocks the translation of cellular mRNAs, this nonstructural protein is not needed for the translation of viral mRNAs (21). More recently, Arnold et al. characterized a rotavirus variant containing a rearrangement in the gene for NSP3. This variant no longer binds efficiently to eIF4G, has a decreased capacity to suppress the translation of cellular mRNAs, and does not induce the accumulation of PABPC in the cell nucleus, yet viral protein synthesis and viral replication are not affected (22), thus supporting the observations that the synthesis of viral proteins is independent of NSP3. Rotavirus-induced inhibition of cell protein synthesis also seems to be mediated by a second mechanism, since eIF2 α becomes phosphorylated early after infection and is maintained in this state throughout the virus replication cycle (20).

Despite the severe inhibitory translation conditions imposed in the host cell by the infection, the viral mRNAs are efficiently translated. In this work, we characterized the relevance of the nuclear accumulation of PABPC and the consequences of this relocalization for the translation of cellular mRNAs in rotavirus-infected cells. Taken together, our results suggest that the shutoff of cell protein synthesis during the infection is a multifactorial process, where the inhibition of transport of cellular mRNAs from the nucleus to the cytoplasm seems to be the most determinant factor in preventing the translation of poly(A)-containing mRNAs and thus the antiviral and stress responses of the cell.

MATERIALS AND METHODS

Cells, virus, and antibodies. The rhesus monkey epithelial cell line MA104 was grown in advanced medium (Dulbecco's modified Eagle's medium [DMEM]—reduced serum) (HyClone, Logan, UT) supplemented with 5% fetal bovine serum (FBS) and was used for all experiments carried out in this work. Rhesus rotavirus (RRV) was obtained from H. B. Greenberg (Stanford University, Stanford, CA). The virus was propagated in MA104 cells as described previously (23). Prior to the infection, RRV was activated with trypsin (10 μ g/ml; Gibco, Life Technologies, Carlsbad, CA) for 30 min at 37°C. Rabbit polyclonal sera to NSP2 and to NSP3 have been described previously (24). Polyclonal antibodies to purified RRV triple-layered particles (TLPs) and to vimentin were produced in rabbits as described previously (25). Monoclonal antibody to PABPC and polyclonal antibody to eIF4GI were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The polyclonal antibody to the C-terminal region of eIF4GII was a gift from N. Sonenberg (McGill University, Montreal, Canada); Alexa Fluor 488- and 647-conjugated secondary antibodies were purchased from Molecular Probes (Eugene, OR). Horseradish peroxidase-conjugated goat anti-rabbit polyclonal antibody was from PerkinElmer Life Sciences (Boston, MA), and streptavidin coupled to horseradish peroxidase and horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin G were from Zymed (San Francisco, CA).

Immunofluorescence. MA104 cells grown in coverslips were infected or not with rotavirus RRV. At different times postinfection, cells were fixed with 2% paraformaldehyde for 20 min at room temperature, and the coverslips were washed four times with phosphate-buffered saline (PBS) with 50 mM ammonium chloride. The fixed cells were permeabilized by incubation in 0.5% Triton X-100 in PBS with 50 mM ammonium chloride and 1% bovine serum albumin (BSA) and then were blocked by incubation with 1% BSA and 50 mM NH₄Cl in PBS at 4°C overnight. The coverslips were incubated with primary antibodies for 1 h at room temperature, followed by incubation with the corresponding secondary antibody for 1 h at room temperature. Coverslips were mounted on glass slides using Fluoprep medium (bioMérieux, Marcy l'Etoile, France). The slides

were analyzed using an E600 epifluorescence microscope coupled to a DXM1200 digital still camera (Nikon, Tokyo, Japan).

Immunoblot analysis. Cells were lysed for 15 min at 4°C in lysis buffer containing 25 mM NaF, 1 mM sodium orthovanadate, 50 mM Tris (pH 7.4), 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and 1% Triton X-100, supplemented with a protease inhibitor cocktail (Complete, EDTA free; Roche, Basel, Switzerland). The lysates were centrifuged for 5 min at 10,000 \times g, and the supernatants were collected. Samples were diluted in Laemmli sample buffer, denatured by boiling for 5 min, subjected to 10%-sodium dodecyl sulfate-7.5% polyacrylamide gel electrophoresis (SDS-7.5% PAGE), and transferred to nitrocellulose membranes (Millipore, Bedford, MA). The membranes were blocked by incubation with 5% nonfat dry milk in PBS for 1 h at room temperature and with primary antibodies diluted in PBS containing 5% milk, followed by an incubation with secondary, species-specific, horseradish peroxidase-conjugated antibodies. The peroxidase activity was developed using the Western Lightning chemiluminescence reagent Plus (PerkinElmer Life Sciences, Boston, MA), following the manufacturer's instructions.

FISH of poly(A)-containing RNAs. To detect the subcellular localization of the polyadenylated mRNAs, the protocol of fluorescence *in situ* hybridization (FISH) described by Mingle et al. (26) was followed. Briefly, MA104 cells were grown in coverslips and infected with RRV at a multiplicity of infection (MOI) of 3. At 6 h postinfection (hpi), cells were fixed with 2% paraformaldehyde in PBS for 15 min at room temperature, washed twice with 50 mM NH₄Cl in PBS, permeabilized with 0.5% Triton X-100, and incubated for 30 min at room temperature with 1.5% H₂O₂. Fixed cells were incubated overnight at 37°C with 120 μ l (0.2 ng/ μ l) of a biotin-labeled oligo(dT) probe in hybridization buffer (5 \times SSC [0.75 M NaCl and 0.075 M sodium citrate], 20 mg/ml heparin, 0.1% Tween 20, and 1 μ g/ml of total yeast RNA). Then, the cells were washed three times with hybridization buffer at 37°C and three times with 0.1 \times SSC and blocked with blocking buffer (1% BSA, 0.2% nonfat milk, and 0.3% Triton X-100) for 1 h at room temperature. The RNA probe was detected by incubating the cells with streptavidin conjugated to peroxidase for 1 h at room temperature. After this step, the fluorescence signal for RNA detection was obtained by using the tyramide signal amplification (TSA) system (TSA-Plus fluorescence palette system; PerkinElmer, Boston, MA) as recommended by the manufacturer. For simultaneous protein immunodetection, specific antibodies were added during the streptavidin incubation (all antibodies were diluted 1:1,000); cells were washed five times with TNT buffer (0.1 M Tris [pH 7.5], 0.15 M NaCl, and 0.05% Tween 20), incubated with the corresponding secondary antibody for 1 h at 37°C, and washed five times with TNT buffer. Finally, the cells were mounted on glass slides with Fluoprep medium (bioMérieux, Marcy l'Etoile, France), and the slides were analyzed using an E600 epifluorescence microscope coupled to a DXM1200 digital still camera (Nikon, Tokyo, Japan).

siRNA transfection. The small interfering RNA (siRNA) directed to eIF4GII had the sequence GGUCCUUUUUAUCCUGGAC (sense) and GUCCAGGAUAAAAAGGACC (antisense), corresponding to nucleotides 533 to 551 of the human eIF4GII gene (GenBank accession number NM_003760). The siRNAs to eIF4GI and to NSP3 used in this work were previously described (21). As an irrelevant control (siRNAIrre), an siRNA directed to the green fluorescent protein was used. All siRNAs were purchased from Dharmacon Research (Lafayette, CO). Transfection of the siRNAs into MA104 cells was performed as previously described (25). Briefly, 2 μ l of Lipofectamine (Invitrogen, Life Technologies, Carlsbad, CA) was added to 100 μ l of 600-pmol/ml siRNAs in minimal essential medium (MEM) without antibiotics and incubated for 20 min at room temperature. The transfection mixture was then added to confluent MA104 cell monolayers previously washed with MEM and incubated for 8 h at 37°C. After this incubation period, the transfection mixture was removed, and the cells were kept in DMEM for 48 h at 37°C prior to infection.

Transfection of siRNA to NSP3 was performed using a reverse transfection method described previously (27). Briefly, a mixture containing

TABLE 1 Oligonucleotides used in this work

Oligonucleotide	Sequence (5'–3')
KasT7-5'fw	AGC AGA TCT AGG ACC AAG CT
5ATGSpeRev	GCG ACT AGT CAT GTT GAA GAC TTC GTT
5'lucSpeI	ACG CAC TAG TGA AGA CGC CAA AAA C
3'lucBglII	GGA AGA TCT ACA ATT TGG ACT TTC CG
VP63'fw	AGC AGA TCT AGG ACC AAG CT
YM6-3'	CAG ACC CGG GCC GCG GTC ACA TCC TCT CAC T
Kas/T7/Gluc	CGC GGC GCC TAA TAC GAC TCA CTA TAG GCT TGG TAC
Gluc/MfeI	GAA TGC AAT TGT TGT TGT TAC
Gluc Forward	CAC GCC CAA GAT GAA GAA GT
Gluc Reverse	TTG AAC CCA GGA ATC TCA GG

the Oligofectamine reagent (15 μ l/ml) (Invitrogen, Life Technologies, Carlsbad, CA) and 200 pmol/ml of the siRNA was added to wells of 48-well plates and incubated for 20 min at room temperature. After this period, 200 μ l of a single cell suspension of 1.5×10^5 MA104 cells/ml was added to each well and incubated for 48 h at 37°C before being infected.

mRNA transfection. *In vitro*-transcribed mRNAs were lipofected into MA104 cells by incubating cell monolayers with MEM containing Lipofectamine 2000 (40 μ g/ml) (Invitrogen, Life Technologies, Carlsbad, CA) and 2.5 μ g/ml of mRNA for 2 h at 37°C. After this time, the transfection mixture was removed, and the cells were washed four times with MEM and incubated for 30 min at 37°C with 10 mg/ml RNase A in MEM to remove the extracellular nonspecifically bound RNA. Then, the cells were washed four times with 1% BSA in MEM and four times with MEM and incubated at 37°C for different times. Rotavirus infection of transfected cells was done by immediately adding virus to the previously washed cells.

Plasmid transfection. The pTK-GLuc plasmid, which has the coding sequence for *Gaussia* luciferase (Gluc), was obtained from New England BioLabs (Ipswich, MA). The plasmid was transfected into confluent cell monolayers using Lipofectamine LTX (Invitrogen, Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Briefly, MA104 cells were incubated for 3 h at 37°C with a mixture of Lipofectamine LTX (40 μ g/ml) and 7.5 μ g/ml of the plasmid. After this time, the transfection mixture was replaced by MEM, and cells were incubated for different times before being infected or harvested.

Luciferase assays. MA104 cells transfected with the indicated mRNAs expressing *Renilla* or firefly luciferases were lysed at different times, and the cellular lysates were divided into two fractions: one fraction was used to extract RNA with TRIzol reagent (Invitrogen, Life Technologies, Carlsbad, CA) according to the manufacturer's instructions, and the other fraction was assayed in duplicate using the Dual-Luciferase reporter assay system (Promega, Madison, WI) for firefly and *Renilla* luciferase activities. For cells expressing *Gaussia* luciferase, the cell medium was used to measure the activity of this protein using the BioLux *Gaussia* luciferase assay kit (New England BioLabs, Ipswich, MA), according to the manufacturer's instructions, while the cells were used to extract RNA using the TRIzol reagent. Luminescence was measured in the Monolight 2010 apparatus (Analytical Luminescence Laboratory, Ann Arbor, MI).

Plasmid constructions. Plasmid pGEM-vFv was constructed as follows; pGEM3Z (Promega) was used as a backbone to introduce, at the KasI and SmaI sites, the firefly luciferase open reading frame (ORF) from pGL3 Basic (Promega) flanked by the 5' and 3' untranslated regions (UTRs) of RRV gene segment 6. Oligonucleotides KasT7-5'fw and 5ATG-SpeRev (Table 1) were used to introduce the 5'VP6 UTR preceded by a T7 promoter and ending at a SpeI site next to the ATG of the firefly *luc* gene. The firefly *luc* ORF was amplified using oligonucleotides 5'lucSpeI and 3'lucBglII (Table 1), which introduced SpeI and BglII sites at the 5' end next to the ATG and a BglII site after the stop codon, respectively, of the firefly ORF; the 3' UTR of gene segment 6 was amplified by PCR using

oligonucleotides VP63'fw and YM6-3' (Table 1), which introduced BglII and SmaI sites at the 5' and 3' ends, respectively.

Plasmid pBF-cRc was constructed by subcloning the *Renilla* luciferase ORF digested from plasmid pRL-CMV (Promega) with XbaI, treating the linearized plasmid with T4DNA polymerase to blunt end the terminal overhangs, and then digesting the plasmid with NheI. After gel purification, the fragment was ligated to the pOO2 expression vector (kindly provided by O. Pantoja, Instituto de Biotecnología, UNAM, Mexico City, Mexico), which contains the 5' and 3' UTRs of the *Xenopus laevis* β -globin gene (GenBank accession number NM_001096347.1), previously digested with XbaI and PvuII. In plasmid pOO2, the 5' UTR of the globin gene is preceded by an SP6 promoter, and at the end of the 3' UTR, it contains a stretch of 60 adenines followed by an MfeI site. All constructs were verified by sequencing.

***In vitro* RNA transcription.** To obtain vFv and rotavirus gene 10 capped mRNAs, plasmids pGEM-vFv and pGEM-NSP4 (28) were linearized with SacII (New England BioLabs, Ipswich, MA) and purified by phenol-chloroform extraction and ethanol precipitation. The linearized plasmids were used as templates for *in vitro* transcription using the MEGAscript T7 kit (Ambion, Life Technologies, Carlsbad, CA) according to the instructions of the manufacturer, in the presence of a cap analog. The T7 polymerization mixture contained 1 μ g of linearized DNA, 7.5 mM (each) ATP, CTP, and UTP, 1.5 mM GTP, and 6 mM cap analog (New England BioLabs). Similarly, mRNA from plasmid pBF-cRc was obtained by *in vitro* transcription of the MfeI-linearized plasmid using the MEGAscript SP6 kit (Ambion, Life Technologies, Carlsbad, CA) according to the instructions of the manufacturer, in the presence of a cap analog. To *in vitro* transcribe the Gluc coding region, oligonucleotides Kas/T7/Gluc and Gluc/MfeI (sequences in Table 1) were used to PCR amplify the Gluc ORF and to introduce a T7 promoter at the 5' end and a MfeI site at the 3' end. The PCR product was digested with MfeI, treated with proteinase K (2 mg/ml), and phenol and chloroform extracted. The purified PCR product was used as a template for *in vitro* transcription using the MEGAscript T7 kit (Ambion, Life Technologies, Carlsbad, CA), in the presence of a cap analog as previously mentioned. After transcription, the Gluc mRNA was *in vitro* polyadenylated using *Escherichia Coli* poly(A) polymerase (New England BioLabs) for 20 min at 37°C. The increase in size of the polyadenylated transcript was verified by running the mRNA in a 1% agarose gel in Tris-acetate-EDTA (TAE) buffer.

Real-time RT-PCR analysis. Purified total RNA extracted from cell lysates was treated with RNA-free DNase (Roche, Basel, Switzerland) to eliminate possible DNA contamination. *Gaussia* luciferase mRNA was amplified using the primers Gluc forward and Gluc reverse (Table 1). The primers used to amplify *grp78*, *grp94*, firefly luciferase, *Renilla* luciferase, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs have been described previously (28–30). The level of each mRNA was determined by one-step real-time quantitative reverse transcription-PCR (qRT-PCR) as previously reported (29). Quantitative analysis of data was performed using the Prism 7000 analysis software program (Applied Biosystems, Life Technologies, Carlsbad, CA). The results were normalized to the levels of total GAPDH mRNA detected in each RNA sample. The changes in gene expression were calculated by the $2^{-\Delta\Delta CT}$ method, where C_T is the threshold cycle (31).

Northern blotting. Total cellular RNA was isolated for Northern blotting using the TRIzol reagent, resolved on 0.8% agarose-formaldehyde gels, transferred to Hybond-N⁺ membranes (Amersham, GE Healthcare, Buckinghamshire, United Kingdom), and probed with a ³²P-labeled *grp78* DNA oligonucleotide, which was labeled using T4 polynucleotide kinase (New England Biolabs, Ipswich, MA). RNase H digestions were carried out with 10 to 13 μ g of total RNA combined with 500 pmol of the oligo(dT)₁₅ primer. After incubation at 65°C for 8 min, 1 U of RNase H (New England Biolabs, Ipswich, MA) and 40 U of RNasin (Promega, Madison, WI) were added to 1 \times RNase H buffer, followed by incubation at 37°C for 30 min. The RNA was extracted using the TRIzol reagent and

subjected to gel electrophoresis. Northern blots were analyzed using a Typhoon 8600 phosphorimager.

Viral mRNA quantitation. Confluent MA104 cells in 48-well plates were infected with RRV and harvested at different time points using the TRIzol reagent. Total RNA was purified and treated with RNA-free DNase (Roche, Basel, Switzerland) to eliminate possible DNA contamination. The primers used for the amplification of gene 10 of rotavirus have been described previously (28). To determine specifically the levels of each RNA strand, RT-PCR was performed, separating the reverse transcription and PCR steps, as described by Ayala-Breton et al. (28). In each assay, a standard curve was generated by amplifying known amounts of *in vitro*-transcribed rotavirus gene 10 mRNA using the reverse primer during the RT step. The quantitative analysis of data was performed using the ABI Prism 7000 analysis software program. After PCR amplification, the ABI Prism software was used to set a cutoff line with the obtained fluorescence values (y axis) for all the samples between the logarithmic phases of the amplification curves. Then, the logarithm of the concentration of each sample was plotted against the cycle number, where the amplification curve of the sample reached the cutoff line (C_T). The amount of positive- or negative-strand RNA from unknown samples was determined by extrapolating the C_T value onto the corresponding standard curve.

Preparation of cytoplasmic and nuclear extracts. Confluent MA104 cells were harvested by trypsinization, washed twice with PBS, and collected by centrifugation. Cells were resuspended and lysed by incubation in buffer 1 (25 mM HEPES, pH 7.9, 5 mM KCl, 0.5 mM MgCl₂, 1 mM dithiothreitol [DTT], and 0.5% [vol/vol] NP-40 supplemented with a protease inhibitor cocktail, Complete [Roche]) for 15 min at 4°C. Nuclei were harvested by centrifugation (5,000 rpm, 5 min), and the resulting supernatant was collected as cytoplasmic extract. The pelleted nuclei were washed once in buffer 2 (25 mM HEPES, pH 7.9, 5 mM KCl, 0.5 mM MgCl₂, 1 mM DTT, and 0.25% [vol/vol] NP-40 supplemented with a protease inhibitor cocktail). Total RNA was extracted from cytoplasmic and nuclear fractions using the TRIzol reagent.

Extraction of polyadenylated mRNAs. After total RNA was extracted from cytoplasmic and nuclear fractions as described above, polyadenylated mRNAs were isolated using the Oligotex Direct mRNA minikit (Qiagen, Germantown, MD) as described by the manufacturer, and the concentration of eluted poly(A)-containing RNA was determined by measuring the absorbance at 260 nm in an ND-1000 spectrophotometer (NanoDrop; Thermo Fisher Scientific, Wilmington, DE).

RESULTS

Rotavirus infection induces the accumulation of poly(A)-containing mRNAs in the nucleus. It has been previously shown that during rotavirus infection, PABPC accumulates in the cell nucleus (19, 20), and this accumulation depends on the presence of NSP3. To determine if the localization of poly(A)-containing mRNAs was also modified in rotavirus-infected cells, we performed a fluorescent *in situ* hybridization (FISH) assay to visualize the cellular localization of the polyadenylated mRNAs. In these experiments, MA104 cells were infected or not with RRV, fixed, and hybridized with a biotin-labeled oligo(dT) probe, complementary to the poly(A) tail; cells were also treated with an siRNA directed to NSP3 or with a control, irrelevant siRNA directed to GFP to establish if the nuclear accumulation of poly(A)-containing mRNAs was related to the presence or absence of this nonstructural protein. Cells were also immunostained with antibodies directed to NSP3 to verify the silencing of this protein during the infection. Figure 1A shows that while the polyadenylated mRNAs were homogeneously distributed in the cytoplasm and in the nucleus of uninfected cells transfected with a control siRNA, the hybridization signal of these mRNAs decreased in the cytoplasm of infected cells but not in the nucleus, where they appear to concentrate. The

signal observed is specific of the hybridization of the biotin-labeled oligo(dT) probe, since we did not detect any fluorescent signal when no probe was added (but peroxidase-conjugated streptavidin and TSA reagent were included). In contrast, when the expression of NSP3 was silenced in infected cells, the distribution of mRNAs with a poly(A) tail appeared very similar to that observed in uninfected cells, indicating that NSP3 is involved in the nuclear accumulation both of PABPC and of poly(A)-containing mRNAs.

It has been previously shown that NSP3 binds to eIF4G at the same place that PABPC does but with higher affinity, evicting it from the eIF4F complex, and it was suggested that this displacement of PABPC from the eIF4F complex by NSP3 during the infection might cause the nuclear accumulation of PABPC; if this were the case, silencing the expression of eIF4GI or eIF4GII or both would have the same effect on PABPC cellular localization. To determine if the relocalization of PABPC to the nucleus is due to its eviction from its binding site in the eIF4F complex, the expression of eIF4GI and that of eIF4GII were silenced, and the effect of these knockdowns on the subcellular localization of PABPC was assessed by immunofluorescence (IF) staining. MA104 cells were transfected with siRNAs directed to eIF4GI, eIF4GII, or a combination of both; 48 h posttransfection (hpt), the cells were infected or mock infected, and 9 h postinfection (hpi), cells were fixed and immunostained with antibodies to NSP2, to PABPC, and to eIF4GI (Fig. 1B); it was not possible to stain eIF4GII in these assays, since the antibody we used did not work for IF, but the knockdown of this protein was determined by Western blotting (Fig. 1C). We found that the distribution of PABPC changed only in rotavirus-infected cells transfected with an irrelevant siRNA. When the expression of eIF4GI, eIF4GII, or a combination of both was silenced, the distribution of PABPC did not change (Fig. 1B). These results suggest that the nuclear accumulation of PABPC is not directly due to its lack of interaction with eIF4GI and/or II but most likely is due to an additional effect caused by NSP3.

The UTRs do not determine the translation efficiency of reporter mRNAs. Once we found that during rotavirus infection the poly(A)-containing mRNAs were accumulated in the nucleus, we decided to characterize the translation efficiency of reporter mRNAs containing either viral or cellular untranslated regions (UTRs) in rotavirus-infected cells. To evaluate this, we constructed transcription plasmids that direct the synthesis of two reporter mRNAs (Fig. 2A). The vFv RNA contains the firefly luciferase ORF flanked by the 23 and 123 nucleotides (nt) of the 5' and 3' UTRs of RRV gene 6, respectively, while the cRc mRNA contains the *Renilla* luciferase ORF flanked by the 19 and 125 nt of the 5' and 3' UTRs of the *Xenopus* globin gene; at the end of the 3' UTR of the globin gene, a stretch of 60 adenines was introduced in the transcription plasmid. *In vitro*-transcribed vFv and cRc mRNAs were transfected into MA104 cells either before or after virus infection or mock infection, and the luciferase activities and the amount of transfected mRNA present under each condition were determined 10 hpt (Fig. 2B). The translation efficiencies of the transfected mRNAs were determined by normalizing the detected luciferase activities with the level of the corresponding mRNA, quantitated by real-time RT-PCR (qRT-PCR) (Fig. 2C). The translation levels of the vFv and cRc mRNAs in infected cells were compared to those in mock-infected cells. It was observed that the translation of both mRNAs was very similar when transfected in

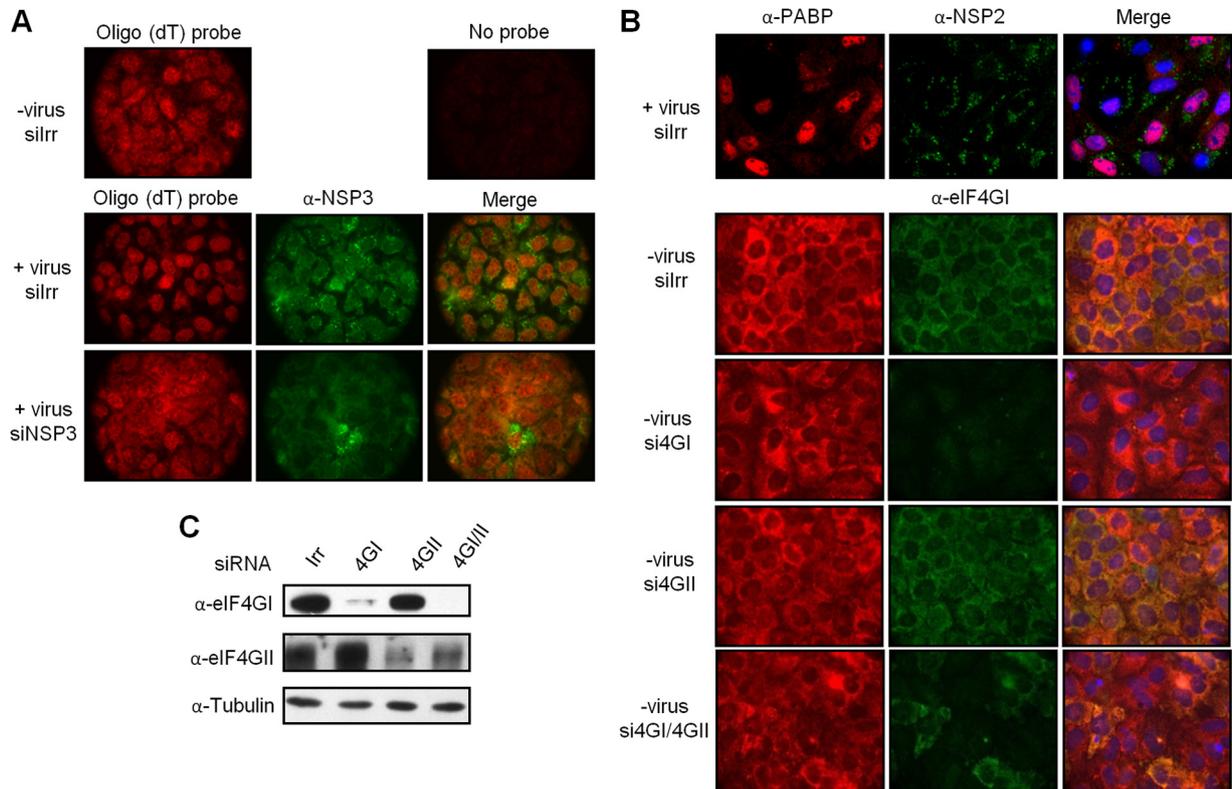


FIG 1 Polyadenylated mRNAs accumulate in the nucleus of infected cells. MA104 cells grown in coverslips were transfected with the indicated siRNAs to NSP3, eIF4GI (si4GI), eIF4GII (si4GII), a combination of both siRNAs (si4GI/4GII), or an irrelevant sequence (silrr); 48 h posttransfection (hpt), the cells were infected with RRV (+virus), or mock infected (-virus), and 9 h postinfection (hpi), the cells were fixed, permeabilized, and hybridized with a biotin-labeled oligo(dT) probe or with no probe and immunostained with anti-NSP3 (A) or immunostained with anti-NSP2, anti-PABPC, and anti-eIF4GI (B), as indicated in Materials and Methods. Proteins or bound probe were detected using the indicated antibodies and secondary Alexa-conjugated antibodies. The fluorescence signal corresponding to the bound probe was developed using a tyramide signal amplification system (TSA) (PerkinElmer). Cell nuclei were stained with 4,6'-diamidino-2-phenylindole (DAPI), shown in blue. (C) In parallel wells, cells were transfected with the indicated siRNAs as for panel B, and 48 hpt, cells were lysed and the proteins were resolved in an SDS-7% PAGE and detected by immunoblot analysis with the indicated antibodies.

cells before the infection. Surprisingly when the reporter mRNAs were transfected in cells that had been previously infected, the translation of both mRNAs was greatly increased; under these conditions the translation of the vFv reporter mRNA was slightly better (although this was not statistically significant) than that of the reporter cRc (Fig. 2C). These results suggest that there is no significant difference in the translation efficiencies of mRNAs containing either viral or cellular UTRs and also suggest that once the cells have been infected, there is a change in the translation machinery that makes it more efficient.

The nucleocytoplasmic transport of reporter mRNAs is blocked in rotavirus-infected cells. We found that a cellular mRNA-like, *in vitro*-transcribed mRNA was efficiently translated when transfected into infected cells; this observation, however, is in conflict with the fact that in rotavirus-infected cells, there is a severe shutoff of cellular protein synthesis. To determine if the efficient translation of transfected mRNAs was due to their direct delivery into the cell's cytoplasm, we compared the translation efficiency of a *Gaussia* luciferase (Gluc) reporter mRNA that was delivered to cells either by transfecting a plasmid that encodes this mRNA or by introducing the mRNA transcribed *in vitro* from the same plasmid. The rationale for this experiment was that the plasmid-encoded Gluc gene is transcribed in the nucleus of the cell, and to be translated it has to be transported to the cell's cytoplasm.

On the other hand, the transfected mRNA will be delivered directly into the cytoplasm, ready for its translation. The *in vitro*-transcribed Gluc mRNA was polyadenylated using poly(A) polymerase to discard differences in the 3' ends of the *in vivo* and *in vitro* transcribed mRNAs. We found that the activity of Gluc expressed from the plasmid in infected cells was about 10-fold less than that in mock-infected cells. In contrast, the amount of Gluc expressed from *in vitro*-transcribed mRNA was about 4 times higher in rotavirus-infected cells than in mock-infected cells (Fig. 3A). To discard differences in mRNA transcription or RNA degradation between these conditions, the activity of *Gaussia* luciferase was normalized to the amount of Gluc mRNA, which was quantitated by qRT-PCR under each condition. To establish if the presence of NSP3 in rotavirus-infected cells was responsible for the reduced activity of Gluc when the reporter was introduced as a plasmid, we silenced the expression of NSP3 by RNA interference (RNAi) (Fig. 3B). We found that when NSP3 was knocked down, the activity of Gluc expressed from the plasmid was very similar to its expression in mock-infected cells, i.e., it seemed that it was no longer inhibited in infected cells, whereas the expression of the *in vitro*-transcribed reporter mRNA was not significantly affected under these conditions. These results suggest that translation of polyadenylated mRNAs is inhibited in rotavirus-infected cells not by a regulation step during the translation process itself but rather

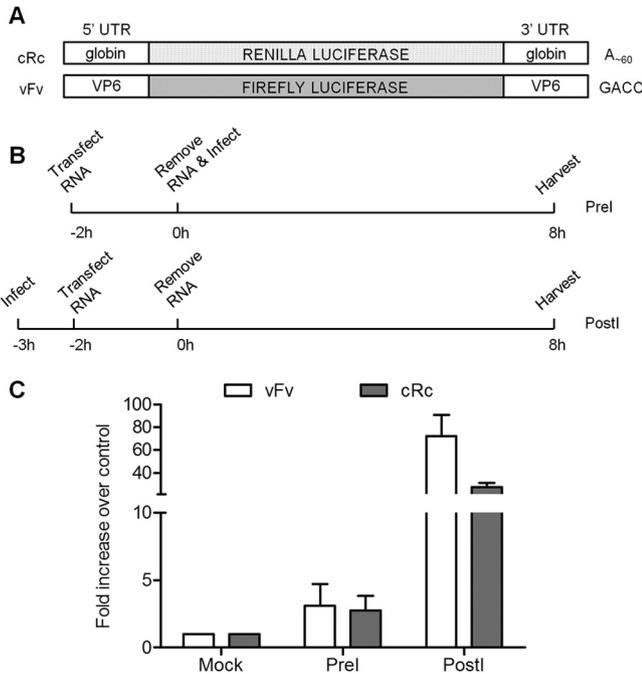


FIG 2 The 5' and 3' UTRs do not determine the translation efficiency of reporter mRNAs. (A) Schematic representation of the chimeric reporter mRNAs cRc and vFv. cRc contains the *Renilla* luciferase ORF flanked by 19 and 125 nt of the globin gene, respectively. The mRNA also contains a tail of poly(A), which was introduced in the template plasmid; vFv contains the firefly luciferase ORF flanked by 23 and 139 nt of the 5' and 3' UTRs, respectively, of RRV gene segment 6. (B) Experimental design for analyzing luciferase synthesis in MA104 cells transfected at two different times relative to the time of infection. cRc or vFv mRNAs were transfected into mock-infected MA104 cells (Mock), 2 h before the infection with RRV rotavirus (PreI), or just after the virus adsorption period (PostI). (C) Ten hours after RNA transfection, the cells were lysed, and the activity of each luciferase was measured and normalized to the amount of the corresponding transfected mRNA that was quantitated in the same lysate by real-time RT-PCR (qRT-PCR). The activities are expressed as a fold increase of the normalized activity obtained when the respective mRNA (cRc or vFv) was transfected in mock-infected cells, which was set as 1. The arithmetic mean \pm SEM for three independent experiments performed in triplicate is shown.

by blocking of the export of mRNAs from the nucleus to the cytoplasm, and NSP3 seems to be directly or indirectly involved in this blockage.

The subcellular localization of cellular mRNAs changes in rotavirus-infected cells. To establish if the inhibition of nucleocytoplasmic transport of mRNAs that we found with the Gluc reporter mRNA also affected endogenous cellular mRNAs, we determined the subcellular distribution of three cellular mRNAs, grp78, grp94, and GAPDH, in infected cells. We have previously reported that during rotavirus infection, some genes of the unfolded protein response (UPR), like grp78, become activated so that an increase of at least 4 times in the amount of this mRNA was observed in infected cells. In contrast, other UPR responsive genes, like grp94, did not increase during infection (30). To determine if these cellular mRNAs changed their distribution in the cell, we studied their distribution in nuclear and cellular fractions in virus-infected cells. We also characterized the distribution of the GAPDH mRNA, which is usually considered a transcript that does not vary significantly in rotavirus-infected cells. In these assays, MA104 cells were infected or mock infected and harvested at

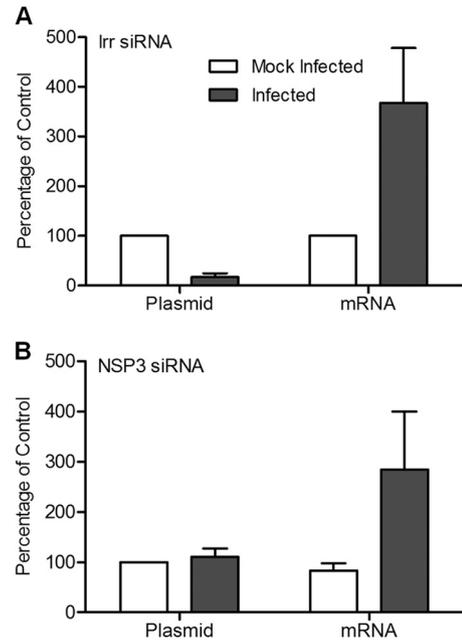


FIG 3 The translation of mRNAs transcribed in the nucleus is inhibited during rotavirus infection. MA104 cells were transfected either with an irrelevant (Irr) siRNA (A) or with an siRNA directed to NSP3 (B) and mock infected or infected with RRV at an MOI of 5. Plasmid pTK-Gluc or *in vitro* transcribed and polyadenylated Gluc mRNA was transfected into MA104 cells just after the virus adsorption period. Cells were harvested 12 hpi, and the *Gussia* luciferase activity was measured in the supernatant of the cell cultures and normalized to the amount of the Gluc mRNA present in the cell lysate under each condition, determined by qRT-PCR. The results are expressed as a percentage of the normalized Gluc activity obtained in mock-infected cells, which was taken as 100%. The arithmetic mean \pm SEM for three independent experiments performed in triplicate is shown.

various times postinfection. The cytoplasmic and nuclear fractions were separated, and the relative amount of each mRNA was determined by qRT-PCR. We found that most of the grp78, grp94, and GAPDH mRNAs were present in the cytoplasmic fraction of mock-infected cells. However, when the grp78 and grp94 mRNAs were quantitated in infected cells at 6 and 12 hpi, we found that there was an increasing amount of the grp78 mRNA in the nuclear fraction and a decrease of the grp94 mRNA in the cytoplasmic fraction. This was not the case for the GAPDH mRNA, which seem to be more concentrated in the cytoplasm, although at 12 hpi there was a small increase, although not significant, in the amount of this mRNA in the nuclear fraction (Fig. 4). To establish whether the change in the cellular localization of the grp78 and grp94 mRNAs was due to rotavirus infection or if this was a generalized event for stressed cells, we characterized the localization of these mRNAs in cells that were treated with thapsigargin, a pharmacological inducer of endoplasmic reticulum (ER) stress. We found that thapsigargin caused an increase in the amount of both grp78 and grp94 mRNAs; however, in contrast with our observations in infected cells, these mRNAs were found mainly in the cytoplasmic fraction.

To determine if NSP3 was involved in the accumulation of the cellular mRNAs in the nuclei of infected cells, we silenced the expression of NSP3, measured the amounts of grp78, grp94, and GAPDH mRNAs by qRT-PCR in nuclear and cytoplasmic fractions, and compared them with their distribution in cells trans-

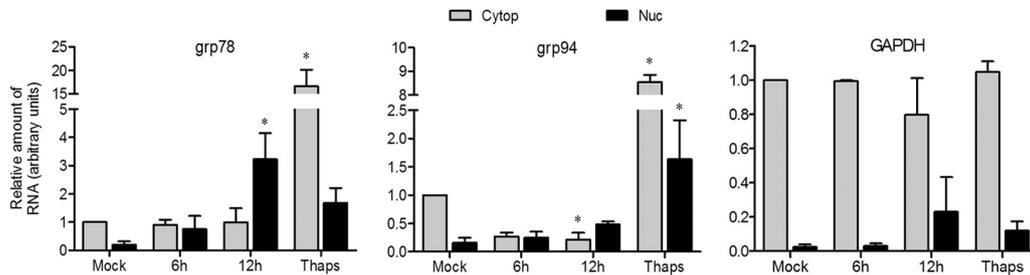


FIG 4 Nucleocytoplasmic distribution of cellular mRNAs in rotavirus-infected cells. MA104 cells were mock infected or infected with rotavirus RRV or were treated with 400 nM thapsigargin (Thaps) for 12 h. At the indicated times postinfection, cells were harvested, and nuclear (Nuc) and cytoplasmic (Cytop) fractions were obtained as described in Materials and Methods. Total RNA was extracted with TRIzol, and grp78, grp94, and GAPDH mRNAs were quantitated in each fraction by qRT-PCR. The qRT-PCR results are expressed as fold increases relative to the amount of each mRNA (grp78, grp94, or GAPDH) present in the cytoplasmic fraction of mock-infected cells, which was taken as 1. The arithmetic mean \pm SEM for three independent experiments performed in triplicate is shown. Asterisks indicate significant differences ($P < 0.05$) between treated cells and their mock counterparts, determined by ANOVA.

ected with an irrelevant siRNA. We found that when NSP3 was silenced, there was an increase in the amounts of grp78 and grp94 mRNAs, as previously observed (30); however, in this case the mRNAs appeared mostly in the cytoplasmic fraction, which was not the case when the cells were transfected with the irrelevant siRNA (Fig. 5). Taken together, these results suggest that during rotavirus infection, there is an accumulation of cellular mRNAs in the nucleus, in which NSP3 plays an important role, and that this differential mRNA distribution is not a general stress response of the cell.

Nuclear accumulation of PABPC induces the hyperadenylation of poly(A)-containing mRNAs. It has been recently found that the nuclear accumulation of PABPC in the nucleus causes the mRNAs to become hyperadenylated and retained in the nucleus (8). Since we found that during rotavirus infection, PABPC and poly(A)-containing mRNAs accumulate in the nucleus we determined if these cellular mRNAs were hyperadenylated. For this, we monitored the length of the grp78 mRNA by using a Northern blot assay. Cells were infected or not with rotavirus, and 12 hpi, total RNA was extracted and hybridized with oligo(dT) and treated with RNase H, and the products were separated by electrophoresis and probed with a 32 P-labeled grp78 DNA oligonucleotide. In rotavirus-infected cells, the length of the grp78 mRNA was extended in a heterogeneous manner compared to that of

mock-infected cells (Fig. 6A). This difference in size must be the result of hyperadenylation, since removal of the poly(A) tail hybridized to oligo(dT) by RNase H digestion resulted in the comigration of the grp78 mRNAs from infected and mock-infected cells. These results suggest that the mRNAs become hyperadenylated during the infection, most likely due to the relocalization of PABPC in the cell nucleus.

The cytoplasm of rotavirus-infected cells has a low abundance of poly(A)-containing mRNAs and contains a high level of viral mRNAs. Once we established that some cellular mRNAs were accumulating in the nuclei of infected cells, we explored if there was also a general decrease of polyadenylated mRNAs in the cytoplasm. For this, MA104 cells were infected or mock infected and harvested at 12 hpi, and the cytoplasmic and nuclear fractions were separated. Poly(A)-containing mRNAs were purified from each fraction by affinity chromatography, and their concentration was measured by spectrometry. We found that there was approximately 50% less poly(A)-containing mRNA in the cytosolic fraction of rotavirus-infected cells than in mock-infected cells (Fig. 6B), while the levels of polyadenylated mRNAs in the nucleus were very similar between infected and mock-infected cells. We also determined the relative abundances of viral mRNAs at different times postinfection. For this, the levels of mRNAs from rotavirus gene segments 6 and 10 were determined by qRT-PCR in

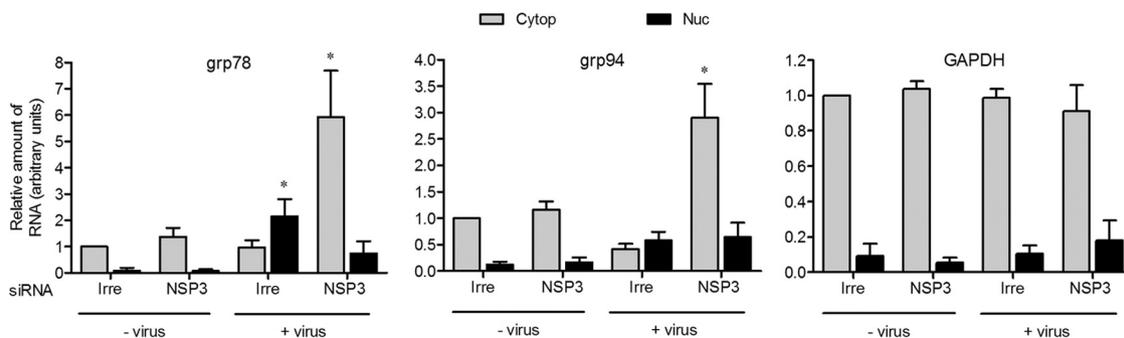


FIG 5 The viral protein NSP3 alters the nucleocytoplasmic distribution of cellular mRNAs in rotavirus-infected cells. MA104 cells were transfected with the indicated siRNAs; 40 h posttransfection, cells were either infected (+virus) or mock infected (-virus), and 12 hpi, cells were harvested, and nuclear (Nuc) and cytoplasmic (Cytop) fractions were obtained as described in Materials and Methods. Total RNA was extracted with TRIzol, and grp78, grp94, and GAPDH mRNAs were quantitated in each fraction by qRT-PCR. The qRT-PCR results are expressed as a fold increase relative to the amount of each mRNA (grp78, grp94, or GAPDH) present in the cytoplasmic fraction of mock-infected cells, transfected with the control siRNA, which was taken as 1. The arithmetic mean \pm SEM for three independent experiments performed in triplicate is shown. Asterisks indicate significant differences ($P < 0.05$) between treated cells and their mock counterparts, determined by ANOVA.

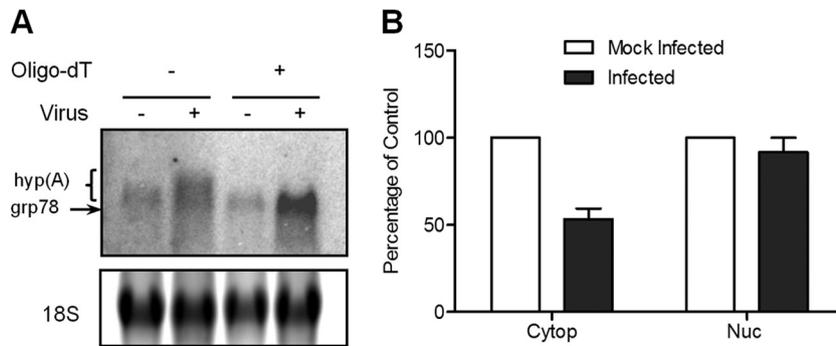


FIG 6 Cellular mRNAs become hyperadenylated, and the amount of poly(A)-containing mRNAs decreases in the cytoplasm of infected cells. (A) MA104 cells were infected or mock infected with rotavirus RRV, and at 12 hpi, the cells were lysed. Total RNA was extracted with TRIzol, hybridized or not with oligo(dT), and digested with RNase H as described in Materials and Methods. The digested products were resolved on a 0.8% agarose-formaldehyde gel, blotted, and hybridized to a ^{32}P -labeled grp78 probe. Hyperadenylated species are indicated by the open bracket [hyp(A) grp78]. The ribosomal 18S rRNA, which was stained with ethidium bromide, is shown as a loading control. (B) MA104 cells were mock infected or infected with rotavirus RRV, and at 12 hpi, total, nuclear, and cytoplasmic fractions were obtained as described in Materials and Methods. RNA was extracted with TRIzol, and poly(A)-containing mRNAs were purified by affinity chromatography using an Oligotex Direct mRNA minikit (Qiagen). The mRNA content of each fraction was determined by measuring the absorbance at 260 nm in an ND-1000 spectrophotometer. The amount of polyadenylated mRNAs in mock-infected cells was taken as 100%.

infected cells. As expected, we found that the amount of viral mRNAs increases during infection in such way that by 8 hpi, there are approximately 300,000 copies of the gene 10 mRNA per cell and about 100,000 copies/cell of gene 6. As controls, we measured the amounts of GAPDH mRNA and of 18S rRNA and found that there are approximately 1,500 and 3,800,000 copies/cell of these RNAs, respectively. These results indicate that during infection, the cytoplasmic pool of polyadenylated mRNAs decreases, while the virus transcribes a very large amount of viral mRNAs that can take advantage of the translation machinery of the cell.

DISCUSSION

The regulation of gene expression is exerted at various levels, ranging from the transcription of the mRNA in the nucleus to its processing, transport to the cytoplasm, and translation of the encoded protein. Viruses use several regulation strategies during translation but also in steps prior to it, which include the impairment of mRNA transport from the nucleus to the cytoplasm. For example, adenovirus and influenza virus selectively inhibit the export of host mRNAs to the cytoplasm. Interestingly, some RNA viruses, like poliovirus and vesicular stomatitis virus, which replicate exclusively in the cytoplasm, also induce the inhibition of nucleocytoplasmic trafficking (32–37). Several viruses target PABPC: poliovirus, calicivirus, hepatitis A virus, and HIV-1 and HIV-2 encode proteases that cleave it (38). Additionally, several other viruses change the subcellular localization of this protein; vaccinia virus, for instance, recruits PABPC to viral replication factories, where transcription and translation occur (39). Herpesvirus, bunyavirus, and rotavirus infections result in the accumulation of PABPC in the nucleus (19, 20, 40–43); however, the mechanism of nuclear retention in these cases has not been established yet, although viral proteins have been implicated.

Early during infection, rotavirus causes a shutoff of cell protein synthesis, with the nonstructural viral protein NSP3 playing a pivotal role in this process. NSP3 regulates the translation of cellular poly(A)-containing mRNAs by preventing their interaction with the eIF4F complex and also by changing the intracellular localization of PABPC, causing its accumulation in the nuclei of infected cells (15, 17–20). Interestingly, here we found that poly(A)-con-

taining mRNAs also accumulate in the nuclei of infected cells in an NSP3-dependent manner. The direct interaction of NSP3 with eIF4G and its involvement in the accumulation of PABPC and polyadenylated mRNAs in the cell nucleus clearly have a negative effect on the synthesis of cellular proteins.

The results obtained in this work suggest that besides a direct inhibitory translation mechanism or in addition to it, during rotavirus infection the translation of poly(A)-containing mRNAs is prevented by blocking their exit from the nucleus. This blockage is related to the presence of NSP3, but the direct mechanism by which this protein affects the nucleus-cytoplasm shuttling of PABPC and of poly(A)-containing mRNAs is not clear. NSP3 does not directly interact with PABPC (17), nor has it been observed in the cell nucleus. Silencing the expression of eIF4GI, eIF4GII, or both did not cause the accumulation of PABPC in the nucleus, suggesting that the eviction of PABPC from its binding site in eIF4G by NSP3 is not the main cause for the relocalization observed in rotavirus-infected cells. Harb et al. found that the interaction of NSP3 with RoXaN might be involved in the relocalization of PABPC (19). However, the direct mechanism by which PABPC is accumulated in the nucleus has not been described yet.

The existence of a translation enhancer in the 3' UTR of rotavirus mRNAs that consists of the terminal sequence 5'-GACC-3' was previously reported (44). These observations were obtained by transfecting rotavirus-infected cells with chimeric mRNAs containing the firefly luciferase ORF flanked either by the 5' and 3' UTRs from rotavirus SA11 gene 6 or by nonviral mRNA UTRs (44). In contrast, in this work we compared the translation of chimeric luciferase reporters containing the 5' and 3' ends of a viral gene versus a chimeric reporter gene containing the UTRs of a cellular gene, including a poly(A) tail. Our results indicate that the UTRs of rotavirus did not give a translational advantage over the UTRs of a cellular mRNA, since the translation of a "viral-like" reporter mRNA was not significantly better than that of a "cellular-like" reporter, even in infected cells. These results suggest that rotavirus mRNAs are preferentially translated over host mRNAs through a mechanism that appears to be independent from *cis*-acting sequences that could be present in their UTRs. Instead, we observed that if the mRNAs were delivered in the cytoplasm of

infected cells, the mRNAs (either viral or cellular) were more efficiently translated than when transfected before viral infection. The increased translation under these conditions of a reporter mRNA with cellular UTRs and a poly(A) tail indicates that mRNAs delivered into the cytoplasm of infected cells are resistant to rotavirus-induced protein synthesis shutoff. With these experiments, however, we cannot rule out the possibility that regions within the viral ORFs might contribute to the translation efficiency of viral mRNAs.

These observations were extended by comparing the translation of a Gluc reporter mRNA delivered into the cell either as a plasmid that needed to be transcribed in the cell nucleus, exported, and then translated versus the same Gluc reporter which was transfected into the cells as a polyadenylated mRNA. The results obtained in these experiments indicate that in rotavirus-infected cells, the translation seems to be regulated mainly by the nucleocytoplasmic transport of mRNAs, indirectly controlled by NSP3; the resistance of the Gluc reporter mRNA to rotavirus-induced shutoff of protein synthesis when transfected after virus infection also confirms that the efficient translation of rotavirus mRNAs is not sequence dependent. These results were further confirmed by analyzing the expression and subcellular localization of three cellular polyadenylated mRNAs (GAPDH, *gpr78*, and *gpr94*) in rotavirus-infected cells. Altogether, these results show that during rotavirus infection, there is a retention of poly(A)-containing mRNAs in the nucleus, with a concomitant reduction in the cytoplasm, that makes them spatially unavailable to be translated.

PABPC is a nucleocytoplasmic shuttling protein (45), although its steady-state localization is cytoplasmic. It was recently reported that increasing the nuclear abundance of PABPC drives hyperadenylation and nuclear retention of mRNAs, thereby inhibiting their expression (8). Interestingly, these authors demonstrated that several divergent viral proteins that inhibit host translation induce the nuclear accumulation of PABP and the hyperadenylation of transcripts in the nucleus (8); furthermore, besides viral infection, other types of stress, like heat shock or oxidative stress, also cause the same phenotype, and thus it has been proposed that controlling the localization of PABPC might be a novel mechanism to globally regulate gene expression (46). Here we also found that during rotavirus infection, host mRNAs became hyperadenylated and retained in the nucleus, most likely due to the nuclear accumulation of PABPC. Since rotavirus mRNAs are transcribed in the cytoplasm, the nuclear accumulation of poly(A)-containing mRNAs would be an effective means to selectively block nascent cellular gene expression, suppressing cellular antiviral responses and avoiding competition for the cell protein synthesis machinery.

In addition to preventing the exit of poly(A)-containing mRNAs from the cell nucleus, the virus transcription machinery directs the synthesis of large amounts of viral mRNAs. We found that the amount of viral mRNAs is in the range of tens of thousands of molecules per cell, which is about 100 times higher than the amount of a typical cellular mRNA (GAPDH) and about 10 times lower than the amount of the 18S rRNA; this large number of mRNAs (of very low complexity, since they represent only 11 different mRNAs) in a cell where the translation of poly(A)-containing mRNAs is inhibited by at least three different mechanisms [eIF2 α phosphorylation, NSP3 evicting PABPC from eIF4G, and poly(A) mRNAs retained in the nucleus] leaves the translation of

viral mRNAs with little or no competition for the protein synthesis machinery and explains the severe shutoff host translation caused by rotaviruses. Indeed, the amount of viral mRNAs produced in an infected cell is so large that we have observed that silencing the expression of the RNA polymerase VP1 or the guanylyl-transferase (VP3) by RNAi results in a decrease of about 90% in the amount of viral mRNAs in the infected cell, yet the amount of viral protein synthesized under these conditions is not decreased, implying that 10% of the viral mRNAs is sufficient to direct the synthesis of all the viral protein needed during the replication cycle of the virus (28).

In summary, our results show that the regulation of gene expression in cells infected with rotavirus seems to be a multifactorial process. At early times of infection, there is an alteration in the host translation machinery in which eIF2 α becomes phosphorylated and NSP3 evicts PABPC from its binding site in eIF4G in such way that the viral mRNAs are preferentially translated. As the infection proceeds, PABPC is relocalized to the cell nucleus, and the newly transcribed cellular mRNAs become hyperadenylated and are accumulated in the cell nucleus, unable to reach the cytoplasm, making them spatially unavailable for translation. While the population of cellular mRNAs decreases in the cytoplasm during infection, the viral mRNAs are transcribed in the cytoplasm at a high rate, resulting in highly favorable conditions for the translation of viral over cellular mRNAs. At the same time, by preventing the translation of cellular genes, the virus controls the antiviral and stress responses of the host. How cytoplasmic mRNAs can be efficiently translated even though eIF2 α is phosphorylated during the infection remains to be answered.

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