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Rotavirus Glycoprotein NSP4 Is a Modulator of Viral Transcription in the Infected Cell
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The outer shell of the rotavirus triple-layered virion is lost during cell entry, yielding a double-layered particle (DLP) that directs synthesis of viral plus-strand RNAs. The plus-strand RNAs act as templates for synthesis of the segmented double-stranded RNA (dsRNA) genome in viral inclusion bodies (viroplasms). The viral endoplasmic reticulum (ER)-resident glycoprotein NSP4 recruits progeny DLPs formed in viroplasms to the ER, where the particles are converted to triple-layered particles (TLPs) via budding. In this study, we have used short interfering RNAs to probe the role of NSP4 in the viral life cycle. Our analysis showed that knockdown of NSP4 expression had no marked effect on the expression of other viral proteins or on the replication of the dsRNA genome segments. However, NSP4 loss of function suppressed viroplasm maturation and caused a maldistribution of nonstructural and structural proteins that normally accumulate in viroplasms. NSP4 loss of function also inhibited formation of packaged virus particles, instead inducing the accumulation of empty particles. Most significant was the observation that NSP4 knockdown led to dramatically increased levels of viral transcription late in the infection cycle. These findings point to a multifaceted role for NSP4 in virus replication, including influencing the development of viroplasms, linking genome packaging with particle assembly, and acting as a modulator of viral transcription. By recruiting transcriptionally active or potentially active DLPs to the ER for conversion to quiescent TLPs, NSP4 acts as a feedback inhibitor down-regulating viral transcription when adequate levels of plus-strand RNAs are available to allow for productive infection.

Consistent with the critical role of the viroplasm in the viral life cycle, knockdown of either NSP2 or NSP5 expression using small interfering RNAs (siRNAs) leads to severely reduced levels of viral RNA and protein synthesis and in the assembly of progeny virus (16, 26). Viroplasms mature from numerous small punctate structures early in infection to fewer, large, highly organized structures at late stages (8). This maturation likely results from the continued accumulation of proteins, RNAs, and newly formed particles in viroplasms and from the fusion of viroplasms with one another.

Two pools of plus-strand RNAs are present in rotavirus-infected cells: one located within viroplasms that serves as templates for dsRNA synthesis and the other located outside of viroplasms that serves as templates for translation (26). These two pools can be resolved based on their susceptibility to RNase degradation by the RNA-induced silencing complexes (RISCs) of the ubiquitous cellular RNA interference system. Specifically, while RISCs cause the degradation of plus-strand RNAs associated with polysomes, they have no detectable impact on plus-strand RNAs that template dsRNA synthesis in viroplasms (26). This phenomenon suggests that the viroplasm functions as a safe house where the viral genome can be packaged and replicated in a microenvironment protected from the antiviral processes of the host. A recent study by Silvestri et al. (26) has indicated that transcriptionally active DLPs present within viroplasms produce the plus-strand RNAs used in genome replication. Such transcriptionally active particles originating from incoming infectious virions may serve as nucleation points for the formation of viroplasms in infected cells (5, 26).

Although direct evidence is lacking, some progeny DLPs formed in viroplasms can be anticipated to amplify the viral replication cycle by supporting secondary rounds of plus-strand RNA transcription. However, most progeny DLPs will migrate...
from viroplasms to the nearby endoplasmic reticulum (ER), where they acquire the outer protein layer of the virion via budding (9). The interaction of DLPs with the ER is mediated by the affinity of the intermediate capsid protein VP6 for the cytosolic tail of the viral ER-transmembrane glycoprotein NSP4 (2, 4, 18, 28). NSP4 exists in association with VP7 in the ER membrane, creating target patches through which DLPs will bud into the lumen of the ER (9). This process yields DLPs contained in the lumen that are transiently ensconced by a lipid envelope. Subsequent loss of the envelope and its NSP4 component allows condensation of VP7 and the assembly of the outer capsid layer. The stage at which VP4 is incorporated into the TLP is not clear, but it may be coincident with budding of the DLP (17) or a later point involving the interaction of VP7-coated DLPs with VP4-containing lipid rafts at the plasma membrane of the infected cell (7).

In previous studies analyzing the role of viral gene products in rotavirus replication using siRNAs (26), we found that knockdown of VP7 expression affected only events down-stream of DLP formation, vis-à-vis assembly of the VP7-VP4 outer capsid layer. Here, we report results of experiments using siRNAs to probe the role of NSP4 in virus replication. In contrast to the expectation that the effects of NSP4 knockdown would mimic that of a VP7 knockdown, our analysis revealed that the loss of NSP4 function has far-ranging effects on the replication cycle, including suppressing the maturation of viroplasms, causing the maldistribution of unassembled capsid proteins, and inducing the assembly of empty particles. However, most striking was the observation that NSP4 knockdown led to dramatically increased levels of viral transcription. These results point to a multifaceted role for NSP4 in the replication cycle, including influencing formation of viral factories and acting as a regulator of viral transcription.

**MATERIALS AND METHODS**

**Cell culture and virus infection.** Fetal rhesus monkey kidney (MA104) cells were maintained in minimal essential medium with Earle’s salts (E-MEM) containing 5% fetal bovine serum (HyClone) and 1% GASP (Quality Biological). Stocks of rotavirus SA11-5N (21) were propagated, and the titers were determined with MA104 cells. Stock suspensions of rotavirus SA11-5N (21) were propagated, and the titers were determined with MA104 cells.

Typically in experiments, MA104 cells were infected with trypsin-activated SA11-5N at a multiplicity of infection of 10. The inoculum was replaced with 80% methionine-free MEM, and 20% E-MEM, containing 25 Ci of \(^{35}S\)-Express Protein Labeling Mix (Perkin-Elmer Life Sciences) per ml. To radiolabel RNA, the inoculum was replaced with 80% methionine-free Dulbecco’s MEM and 20% E-MEM and then supplemented at 3 h p.i. with 25 µCi of \(^{35}S\)-orthophosphate (8,000 to 9,000 Ci/mmol) and 5 µg of actinomycin D per ml.

**siRNA transfection.** Duplex siRNAs specific for sequences within the open reading frame of gene 1 (g1), g2, g9, and g10 of the SA11-5N strain and g5 of the Wa strain were obtained from Dharmacon Research (Table 1). The Wa g5 siRNA is not specific for any sequence of the SA11-5N genome and therefore was used as a control irrelevant (IR) siRNA in transfection experiments of SA11-5N-infected cells. Transfection of siRNA into MA104 cells was performed as previously described (26), using OptiMEM I (Invitrogen) containing 2% Lipofectamine 2000 (Invitrogen) and 0.5 µM siRNA. Cells were infected with rotavirus at approximately 24 h posttransfection.

**Viral protein analysis.** Rotavirus-infected cells were scraped into the medium, pelleted, and resuspended in water containing 1 µg (each) of leupeptin and aprotinin per ml. Lysates were prepared by three cycles of freezing-thawing and typically analyzed under combined denaturing-reducing conditions by electrophoresis on 10% NuPAGE Bis-Tris gels (Invitrogen). In instances where VP7 was to be analyzed by a Western blot assay, samples were not reduced prior to electrophoresis. \(^{35}S\)-Labeled proteins resolved on gels were detected by autoradiography and quantified with a phosphorimager. For Western blot analysis, proteins were transferred from gels onto nitrocellulose membranes, and the blots were soaked in phosphate-buffered saline (PBS) containing 5% milk. Proteins were detected on blots using guinea pig polyclonal antisera prepared against recombinant VP2 (VP2; 1:2,000) (26), rNSP2 (1:11,000) (27), and rNSP5 (1:5,000) and mouse monoclonal antibodies against VP7 (M60; 1:1,000) (25) and NSP4 (B4-1/55; 1:1,000) (22). Horseradish peroxidase-conjugated goat anti-guinea pig and anti-mouse antisera at dilutions of 1:10,000 were used as secondary antibodies. Blots were developed with SuperSignal West Pico Chemiluminescent substrate (Pierce) and exposed to BioMax MR X-ray film.

**Viral RNA analysis.** Rotavirus-infected MA104 cells were transfected with siRNAs, infected with SA11-5N, and radiolabeled with \(^{32}P\)orthophosphate. At the indicated times p.i., the cells were scraped into the medium, pelleted by low-speed centrifugation, washed once with cold phosphate-buffered saline, and lysed by suspension in 100 mM Tris-HCl (pH 8), 150 mM NaCl, 30 mM Na2EDTA, and 1% Triton X-100 for 5 min. After removal of nuclei and larger debris by low-speed centrifugation, the lysates were adjusted to 0.5% sodium dodecyl sulfate and 250 mM NaCl. RNAs were purified from the lysates by phenol:chloroform extraction and ethanol precipitation and analyzed by electrophoresis on 5% polyacrylamide gels containing 7 M urea (26). Radiolabeled RNAs were detected by autoradiography and quantified with a phosphorimager.

**Electrophoretic separation of virus particles.** Intracellular subviral particles were prepared from rotavirus-infected cells as reported earlier, with only minor modification (11). Briefly, MA104 cells were transfected with siRNAs, infected with SA11-5N, and radiolabeled with \(^{35}S\)-labeled amino acids. At 9 h p.i., the cells were washed and resuspended in cold dilute RSB (10 mM Tris-HCl [pH 8.1], 10 mM NaCl, 1.5 mM MgCl2) containing 1 µg (each) of leupeptin and aprotinin per ml. After incubation on ice for 10 min, the cells were disrupted by Dounce homogenization, and the lysates were adjusted to 2% NP-40. Nuclei and larger debris were removed from lysates by low-speed centrifugation. Virus particles were pelleted from the supernatant by centrifugation at 160,000 × g in a Beckman SW40Ti rotor for 2 h, resuspended in TBS buffer (25 mM Tris-HCl [pH 7.4], 137 mM NaCl, 5 mM KCl, 1 mM MgCl2, 0.7 mM CaCl2, 0.7 mM Na2HPO4, 5.5 mM dextrose), and banded in CsCl gradients (20). Fractions were collected and analyzed for acid-precipitable radioactivity by liquid scintillation counting and then dialyzed against 2 mM Tris-HCl (pH 7.5), 0.5 mM Na2EDTA, and 0.5 mM dithiothreitol. Fractions were analyzed for protein content by electrophoresis on 10% NuPAGE gels, followed by autoradiography. Protein quantities were determined with a phosphorimager.

**Electrophoretic separation of virus particles.** Intracellular subviral particles were prepared from rotavirus-infected cells as reported earlier, with only minor modification (11). Briefly, MA104 cells were transfected with siRNAs, infected with SA11-5N, and radiolabeled with \(^{35}S\)-labeled amino acids. At 9 h p.i., the cells were washed and resuspended in cold dilute RSB (10 mM Tris-HCl [pH 8.1], 10 mM NaCl, 1.5 mM MgCl2) containing 1 µg (each) of leupeptin and aprotinin per ml. After incubation on ice for 10 min, the cells were disrupted by Dounce homogenization, and the lysates were adjusted to 2% NP-40. Nuclei and larger debris were removed from lysates by centrifugation at 12,000 × g for 10 min. Clarified lysates were adjusted to a density of 1.365 g/cm3 with CsCl, and the solution was centrifuged at 100,000 × g for 18 h to band virus particles. Fractions from the gradient were dialyzed against low-salt buffer (2 mM Tris-HCl [pH 7.6],

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**TABLE 1. siRNA target sequences**

<table>
<thead>
<tr>
<th>Target gene/protein/strain</th>
<th>siRNA name</th>
<th>Target sequence (nucleotide position)</th>
</tr>
</thead>
<tbody>
<tr>
<td>g1/VP1/SA11-5N</td>
<td>g1-VP1</td>
<td>AAGGAGAUAGACCUAUUGC (510–530)</td>
</tr>
<tr>
<td>g2/VP2/SA11-5N</td>
<td>g2-VP2</td>
<td>ACGGAAUCUCAUGAUGAUCC (2320–2340)</td>
</tr>
<tr>
<td>g9/VP7/SA11-5N</td>
<td>g9-VP7</td>
<td>AAGUGGAAUAACUGAAGGUG (715–735)</td>
</tr>
<tr>
<td>g10/NSP4/SA11-5N</td>
<td>g10-NSP4</td>
<td>AAGUGACUGACAAGUAGGAAG (550–570)</td>
</tr>
<tr>
<td>g5/NSP1/WA</td>
<td>IR</td>
<td>AUCUGCUUUUGGGCGUAGGAUG (782–802)</td>
</tr>
</tbody>
</table>

* Described in Silvestri et al., 2004 (26).
0.5 mM Na2EDTA, 0.5 mM dithiothreitol) and analyzed for particle content by electrophoresis on nondenaturing Tris–glycine–0.6% agarose gels (11). The RNA content of particles was assessed by soaking gels in a solution of ethidium bromide (EtBr). After drying, gels were analyzed for 35S-labeled particles by autoradiography.

Detection of viral proteins by immunofluorescence (IF). MA104 cells were grown to near confluence on glass coverslips, transfected with siRNAs, infected with SA11-5N, and examined by IF as described previously (26). Cells were fixed with 4% paraformaldehyde, permeabilized with 1% Triton X-100, and then incubated with guinea pig polyclonal anti-sera prepared against rNSP2 (1:500) (27) or rVP2 (1:1000) (26) or with mouse monoclonal antibodies against NSP4 (B4-1/55; 1:1,000) (22), VP6 (1026; 1:300) (12), NSP2 (1:1,000), or NSP5 (1:1,000) (23). Anti-guinea pig AlexaFluor 488- and anti-mouse AlexaFluor 594-conjugated antibodies were used as secondary antibodies. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) (Pierce). Fluorescence was detected with a Leica TCS NT confocal microscope. Images were processed with Adobe PhotoShop version 7.0.

RESULTS

Effect of NSP4 and VP7 knockdown on viral protein synthesis. To examine the effect of NSP4 and VP7 knockdown on the expression of viral proteins in rotavirus-infected cells, siRNAs specific for g10 (NSP4) and g9 (VP7) RNAs and an IR siRNA were transfected into MA104 cells (Table 1). The following day, the cells were infected with rotavirus and then maintained in the presence of 35S-labeled amino acids until 9 to 15 h p.i., when the cells were harvested. Viral proteins in lysates recovered from the cells were analyzed by gel electrophoresis and autoradiography and by Western blot assay (Fig. 1). The analysis showed that the NSP4 and VP7 siRNAs were effective in suppressing the expression of NSP4 and VP7, respectively, such that the accumulated levels of these proteins were reduced by 70 to 85% from those that accumulated in cells transfected with the IR siRNA (Fig. 1B and C). Given previous analysis showing that the transfection efficiency of MA104 cells with siRNAs is 80 to 90% (26), the reductions in NSP4 and VP7 expression came close to the maximum values that are theoretically possible for these experiments.

Despite the effective knockdown of NSP4 and VP7 expression in infected cells transfected with the NSP4 and VP7 siRNAs, the expression of the other viral proteins continued at levels approximating that of the IR siRNA-treated cells (Fig. 1). The analysis showed that the NSP4 and VP7 siRNAs were effective in suppressing the expression of NSP4 and VP7, respectively, such that the accumulated levels of these proteins were reduced by 70 to 85% from those that accumulated in cells transfected with the IR siRNA (Fig. 1B and C). Given previous analysis showing that the transfection efficiency of MA104 cells with siRNAs is 80 to 90% (26), the reductions in NSP4 and VP7 expression came close to the maximum values that are theoretically possible for these experiments.

Despite the effective knockdown of NSP4 and VP7 expression in infected cells transfected with the NSP4 and VP7 siRNAs, the expression of the other viral proteins continued at levels approximating that of the IR siRNA-treated cells (Fig. 1). This included the nonstructural proteins needed for viroplasm formation (NSP2 and NSP5) and the structural proteins needed for assembly of cores and DLPs (VP1, VP2, VP3, and VP6). Thus, the effects of the NSP4 and VP7 siRNAs were largely specific to the protein products of the genes to which they were targeted. The in toto similarity in the levels of the 35S-labeled proteins made in cells containing the NSP4 or VP7 siRNA with that of cells containing the IR siRNA indicated that the aggregate level of protein translation in these cells was similar (Fig. 1A). This is in contrast to the across-the-board reduction in viral protein synthesis that occurs when cells are transfected with siRNAs targeting genes encoding NSP2 (g8) (26) or NSP5 (g11) (16).

Enhanced viral transcription correlated with NSP4 knockdown. The effect of NSP4 and VP7 knockdown on viral RNA synthesis was evaluated by preparing cytoplasmic RNA samples from infected cells transfected with the NSP4, VP7, or IR siRNAs and maintained in the presence of 32P orthophosphate from 3 h p.i. until the time of harvest. Radiolabeled plus-strand RNAs and dsRNAs in the samples were resolved by gel electrophoresis and detected by autoradiography (Fig. 2). The analysis indicated that knockdown of NSP4 or VP7 expression had little or no effect on the synthesis of plus-strand RNAs or dsRNAs during the early phase of the replication cycle (3 to 6 h p.i.). However, after that time, the level of transcription occurring in cells in which NSP4 expression was suppressed was severalfold higher than that occurring in cells in which VP7 expression was suppressed or in cells treated with the IR siRNA. Indeed, based on quantification of g11 plus-strand RNAs band intensities, the

FIG. 1. Viral protein expression in NSP4 siRNA-transfected cells. MA104 cells were transfected with the indicated siRNA, infected with SA11-5N, and maintained in medium containing 35S-labeled amino acids. Viral proteins in cell lysates prepared at 9 h (A and B) or 12 h (C) p.i. were analyzed by gel electrophoresis and autoradiography (A) and by Western blot assay using VP2-, VP7-, NSP2-, NSP4-, or NSP5-specific antisera (B and C). The results of the Western blot assay were quantified, and the values were normalized to those for the IR siRNA-treated cell lysates, which were set at 100%.
level of plus-strand RNA synthesis was determined to be 6- to 7-fold higher at 9 h p.i. and 9- to 13-fold higher at 15 h p.i. in the NSP4 siRNA-treated cells than in the VP7 or IR siRNA-treated cells. As is most apparent from analysis of RNA samples collected at 15 h p.i. (Fig. 2), little or no difference was detected in dsRNA synthesis among the cells treated with the NSP4, VP7, or IR siRNAs. Thus, the increased levels of viral transcription resulting from knockdown of NSP4 expression did not result in increased genome replication.

**Regulatory function of NSP4 in viral transcription.** An important function of NSP4 is to recruit DLPs to the ER, where the particles begin a morphogenetic process that leads to the conversion of DLPs to TLPs. Given the role of DLPs as potential transcription machines, it can be assumed that changes in the levels of DLPs in the cell could have an impact on the intracellular levels of plus-strand RNA synthesis. Based on these concepts, we speculated that the increased levels of transcription in infected cells containing the NSP4 siRNA were due to the absence of the necessary NSP4 required to induce the conversion of transcriptionally active DLPs to TLPs. To explore this possibility further, we analyzed the effect of transfecting VP1 and VP2 siRNAs individually or in combination with the NSP4 siRNA on levels of viral transcription in infected cells. Because knockdown of VP1 and VP2 expression would prevent the formation of functional progeny DLPs, levels of plus-strand RNAs accumulating in cells transfected with the VP1 or VP2 siRNAs would be largely dependent on the transcriptase activity of DLPs derived from virions infecting the cells.

Analysis of viral protein expression in the transfected cells showed that the VP1 and VP2 siRNAs were highly effective in knocking down the expression of VP1 and VP2, reducing their expression by >80% (Fig. 3A). Knockdown of either VP1 or VP2 expression by these siRNAs also led to moderate reductions in the expression of other viral proteins. For example, note that a Western blot assay showed that the level of VP6 expression in VP1 or VP2 siRNA-treated cells was reduced by ∼50%. Thus, the expression of both VP1 and VP2 is required in the typical viral replication cycle to achieve maximal levels of overall protein synthesis. Surprisingly though, analysis of cells double transfected with VP1/NSP4 siRNAs or VP2/NSP4 siRNAs showed that the effect of either VP1 or VP2 knockdown on overall protein synthesis was largely overcome when the expression of NSP4 was also suppressed in the infected cell. Again, for example, note that a Western blot assay showed that the level of VP6 expression in the double-transfected cells was at least as great as the level of VP6 expression in the IR, VP1, or VP2 siRNA-treated cells. Collectively, these data indicate that NSP4 loss of function provided conditions allowing increased levels of overall protein synthesis in the infected cell, despite low levels of VP1 or VP2 expression.

To address the possibility that NSP4 knockdown led to higher levels of overall protein synthesis in cells cotransfected with the VP1/NSP4 or VP2/NSP4 siRNAs, 32P-labeled RNAs were recovered at 9 h p.i. from infected cells cotransfected or transfected individually with the siRNAs. Analysis of the RNAs by gel electrophoresis (Fig. 3B) showed that transfection of cells individually with the VP1 or VP2 siRNAs resulted in decreased levels of plus-strand RNA and dsRNA synthesis, compared to cells containing the IR siRNA. However, when the NSP4 siRNA was cotransfected with the VP1 or VP2 siRNA, plus-strand RNA synthesis was restored to levels exceeding that occurring even in the IR siRNA-treated cells (Fig. 3B). Because VP1 or VP2 is essential for formation of functional progeny DLPs, the source of the transcriptase activity responsible for the increased levels of plus-strand RNA synthesis in cells transfected with the VP1/NSP4 or VP2/NSP4 siRNAs was not likely newly assembled DLPs. Instead, the data suggest that DLPs derived from infecting virions were responsible for the increased levels of plus-strand RNA synthesis. Although NSP4 knockdown promoted higher levels of transcription in the cells double transfected with VP1/NSP4 and VP2/NSP4 siRNAs, the levels of transcription did not reach that occurring in cells.
transfected with the NSP4 siRNA alone (Fig. 3B). This too may be connected to the inability of the double-transfected cells to produce transcriptionally active progeny DLPs, in comparison to the infected cell transfected only with the NSP4 siRNA. Nonetheless, the data point to an important role for NSP4 as a regulator of intracellular transcription.

**Effect of NSP4 knockdown on particle formation.** Plaque assays showed that transfection of NSP4 and VP7 siRNAs reduced the assembly of infectious virus to ~13 and 7%, respectively, of that assembled in cells containing the IR siRNA (Fig. 4). To understand the effect of NSP4 and VP7 knockdown on virus assembly, cells were transfected with NSP4, VP7, or IR siRNAs; infected with rotavirus; and maintained in the presence of 35S-labeled amino acids until time of harvest. Virus particles were pelleted from detergent-treated lysates prepared from the cells and banded by CsCl centrifugation. Fractions from the gradients were then analyzed for acid-precipitable radioactivity, density, and protein content to assess the accumulation of TLPs (predominantly banding in fractions 9 to 11), DLPs (fraction 12), and empty particles (fractions 2 to 3) in the cells (Fig. 5). As expected, TLPs were

**FIG. 3.** Enhanced transcription upon NSP4 knockdown. MA104 cells were transfected with the indicated siRNA, infected with SA11-5N, maintained in medium containing 35S-labeled amino acids or [32P]orthophosphate, and harvested at 9 h p.i. (A) Viral proteins in cell lysates were analyzed by gel electrophoresis, autoradiography, and Western blot assay with VP1-, VP2-, VP6-, or NSP4-specific antisera. The results of the Western blot assay were quantified, and the values were normalized to those for the IR siRNA-treated cell lysates, which were set at 100%. (B) RNAs recovered from cell lysates were analyzed by electrophoresis on urea-polyacrylamide gels and autoradiography. Positions of viral plus-strand RNAs (1 to 11) and dsRNAs (ds1 to ds11) are shown.

**FIG. 4.** Effect of NSP4 knockdown on virus titers. Lysates prepared from MA104 cells transfected with the indicated siRNA and infected with SA11-5N were analyzed for virus titer by plaque assay. The results represent the average of three independent experiments, with each evaluated in duplicate. The average titer and standard error are given in parentheses. Values were normalized to 100% for lysates from cells transfected with IR siRNAs.

**FIG. 5.**
the predominant type of particle accumulating in infected cells treated with the control IR siRNA, regardless of the time of harvest (6, 9, or 15 h p.i.). In comparison, the accumulation of TLPs was severely reduced in cells in which VP7 or NSP4 expression was suppressed. Instead, the effect of VP7 knockdown was to cause an increase in the accumulation of DLPs, relative to TLPs, in infected cells (fraction 12 of the VP7 siRNA-treated cells) (Fig. 5). An accumulation of DLPs was less obvious in infected cells transfected with the NSP4 siRNA (compare fractions 12 of the NSP4 and VP7 siRNA-transfected cells at 9 and 15 h p.i.). However, uniquely associated with the NSP4 knockdown was evidence of an increased accu-

FIG. 5. Virus particles in infected cells containing siRNAs. MA104 cells were transfected with the indicated siRNA, infected with SA11-5N, maintained in medium containing 35S-labeled amino acids, and harvested at 6, 9, or 15 h p.i. Virus particles pelleted from the cell lysates were resolved by CsCl centrifugation. Gradient fractions were analyzed for density and radioactivity (A) and for protein content by electrophoresis on 10% NuPAGE gels (B). (A) Black and gray arrowheads indicate positions of TLPs (1.35 g/cm³) and DLPs (1.37 g/cm³) in gradients.
mulation of empty (~1.31-g/cm³) particles at 9 and 15 h.p.i. (fractions 2 and 3 of the NSP4 siRNA-transfected cells).

To evaluate the possibility that NSP4 knockdown led to the accumulation of empty particles, 35S-labeled particles were recovered at 9 h.p.i. from infected cells treated with NSP4 or IR siRNAs. Particles contained in the infected-cell lysates were banded in CsCl gradients, and then resolved into TLP and DLP species by electrophoresis on nondenaturing 0.6% agarose gels (11). Radiolabeled particles in the gel were detected by autoradiography, and particles which contained RNA (i.e., packaged particles) were identified by staining the gel with EtBr (Fig. 6). Positions of TLPs and DLPs in the agarose gels were verified by electrophoretic analysis of the protein content of particles eluted from agarose gels (not shown). The results showed that packaged TLPs and DLPs accumulated in the IR siRNA-treated cells (Fig. 6, fractions 4 to 5 and 6 to 7, respectively), with little evidence of the assembly of empty particles (fraction 1). In contrast, fewer packaged TLPs and DLPs were recovered from the NSP4 siRNA-treated cells. The analysis also revealed evidence of a substantial accumulation of empty DLPs in the NSP4 siRNA-treated cells (Fig. 6, fraction 1), to a level in fact that exceeded the accumulation of packaged DLPs. The level of empty DLPs that formed in the NSP4 siRNA-treated cells also exceeded the assembly of empty TLPs. Together, these data indicate that during the typical course of the viral replication cycle, NSP4 plays a role in preventing the self-assembly of inner capsid proteins into empty particles.

**Role of NSP4 in viroplasm formation and protein distribution.** The impact of NSP4 knockdown on the formation of viroplasms and the intracellular distribution of viral proteins was evaluated by IF staining of infected cells at 9 h.p.i. with a variety of antisera specific for viral structural and nonstructural proteins. The analysis consistently showed that smaller viroplasms were formed in NSP4 siRNA-treated cells than in IR siRNA-treated cells (Fig. 7). In addition, NSP4 knockdown was correlated with a more diffuse appearance of proteins that typically accumulate in viroplasms, including NSP2 and NSP5, proteins that in combination can drive the assembly of viroplasm-like structures, and VP2 and VP6, proteins that form the inner and intermediate shells of DLPs. The overall similarity in the expression of structural proteins (e.g., VP2, and VP6) and nonstructural proteins (e.g., NSP2 and NSP5) in NSP4 and IR siRNA-treated cells (Fig. 1) rules out the possibility that viroplasm development was suppressed in the NSP4 siRNA-treated cells due to decreased viral protein production. Indeed, the similarity in protein levels combined with the differences in numbers and sizes of viroplasms in the NSP4 and IR siRNA-treated cells indicates that the size of the intracellular viral protein pool is not the sole factor governing viroplasm development in vivo. Together, these data provide evidence of a role for NSP4 in promoting interactions between NSP2 and NSP5 required for viroplasm development and for the localization of inner capsid proteins to these inclusions.

**DISCUSSION**

A primary role reported for NSP4 during rotavirus replication is as an intracellular receptor of DLPs, a function necessary for recruitment of these particles to the ER, where they are converted to TLPs. Given this background, we anticipated that knockdown of NSP4 expression would yield a phenotype in the infected cells equivalent to that of a VP7 knockdown: (i) wild-type levels of plus-strand RNA and dsRNA synthesis, (ii) wild-type levels of viral protein synthesis except for the siRNA-targeted gene product, (iii) normal development of viroplasms, and (iv) accumulation of DLPs. Instead, the results showed that knockdown of NSP4 expression resulted in (i) increased
FIG. 7. Viroplasm formation and protein distribution in NSP4 siRNA-treated cells. MA104 cell monolayers were transfected with the indicated siRNAs and infected with SA11-5N. At 9 h p.i., the monolayers were processed for IF assay using the indicated combination of primary antibodies (α) and Alexa-488-conjugated secondary antibodies (green) and Alexa-594-conjugated secondary antibodies (red). Colocalization of signals is indicated by yellow, and cell nuclei were visualized with DAPI (blue).
levels of plus-strand RNA synthesis but wild-type levels of dsRNA synthesis, (ii) underdeveloped viroplasms despite wild-type levels of viral protein synthesis, and (iii) decreased assembly of packaged particles (DLPs and TLPs) and increased assembly of empty particles. These data lead to the conclusion that NSP4 contributes to rotavirus replication at multiple stages, including several that impact events occurring upstream of the step of DLP to TLP conversion.

NSP4 loss of function resulted in increased levels of plus-strand RNA synthesis during later stages of viral infection, regardless of whether infected cells were transfected with only an NSP4 siRNA or cotransfected with an NSP4 siRNA and an siRNA targeting a gene encoding a core protein (i.e., VP1 or VP2). This finding points to a role for NSP4 as a regulator of viral transcription in the infected cell. Specifically, the presence of NSP4 appears to have the impact of limiting plus-strand RNA accumulation in the cell to a level that is much less than is possible in the absence of the protein. Given its DLP-binding activity, NSP4 can be predicted to exert this effect on plus-strand RNA synthesis by interacting with transcriptionally active or potentially active DLPs, causing their conversion to quiescent TLPs.

Because the loss of either VP1 or VP2 function impedes the assembly of functional progeny DLPs in the infected cell, the increased levels of transcription that occurred upon cotransfection of VP1 and NSP4 siRNAs or VP2 and NSP4 siRNAs are not likely to have been due to the activity of progeny DLPs. Instead, the increased levels of transcription into the cotransfected cells must have resulted primarily if not exclusively from the increased transcriptional activity of DLPs originating in the cell from infecting virions. This increased transcriptional activity most likely reflects an increase in the length of time at which virion-derived DLPs remain active in the cell (i.e., increased half-life of activity), as opposed to an increase in the level of polymerase activity associated with each DLP (i.e., increased specific activity). This increased time of activity may be apparent at earlier times (3 to 6 h) of the infection cycle when intracellular events favor RNA amplification. However, at later times (9 to 15 h p.i.), when the infection cycle can be expected to shift to a phase of rapid and efficient conversion of DLPs to TLPs, the absence of NSP4 may result in the accumulation of transcriptionally competent DLPs that then promote abnormally high levels of plus-strand RNA synthesis.

The observation that the intracellular accumulation of plus-strand RNA increases significantly in the presence of an NSP4 siRNA indicates that the level of viral transcription that occurs during the typical infection cycle falls well below that which the DLPs are capable of delivering, at least within the MA104 cell line used in our analyses. Thus, there is an inherent flexibility in the levels of plus-strand RNAs that can be produced in infected cells, potentially providing a means by which the virus can react to variations in the efficiency of viral protein expression that may occur in different cell types or in cells exposed to antiviral factors (e.g., interferon). In essence, NSP4 can be hypothesized to act as a feedback inhibitor in the infected cell that signals to the viral transcription system when adequate plus-strand RNAs have been generated to allow productive infection.

Unlike plus-strand RNA synthesis which increased markedly in the infected cell upon NSP4 knockdown, levels of viral protein synthesis showed little change, if any, barring the NSP4 target protein itself. Because the higher level of transcription did not result in a higher level of translation, we can conclude that the level of viral protein synthesis occurring during the typical infection cycle is at or comes close to the maximum possible. In addition, given that the level of dsRNA synthesis also did not increase in NSP4-siRNA transfected cells, we can rule out the possibility that the level of plus-strand RNA is the sole factor affecting the level of genome replication in the infected cell.

Interestingly, although NSP4 loss of function did not have significant effects on the accumulation of viral proteins in the infected cell, it was correlated with defects in the maturation of viroplasms to the larger-sized inclusions that become prevalent late in the infection cycle. This is remarkable because in infected cells containing an NSP4 siRNA, the levels of the two proteins (NSP2 and NSP5) that drive viroplasm development were the same as those in infected cells containing an IR siRNA. Likewise, the levels of the structural protein known to accumulate in viroplasms (e.g., VP1, VP2, and VP6) were the same in the cells transfected with an NSP4 and IR siRNA. Thus, the failure of viroplasm maturation in the NSP4 siRNA-treated cells apparently was not due to deficiencies in the expression of viroplasmic-resident proteins. Instead, the failure appears to be connected to lack of appropriate translocation of the viroplasm-resident proteins. We suggest from these data that NSP4 plays a critical role in the appropriate maturation of viroplasms by altering the intracellular environment of the infected cell to favor the appropriate transport and accumulation of nonstructural and structural proteins in viroplasms. Certainly, the NSP4 knockdown results are significant in demonstrating that the maturation of viroplasms is driven by more than just the availability of proteins that normally accumulate in such inclusions.

Despite the lack of viroplasm maturation in NSP4 siRNA-treated cells, the level of dsRNA synthesis was similar to that in IR siRNA-treated cells. This result indicates that viroplasm size is not a predictor of levels of dsRNA synthesis occurring within the infected cell and that the smaller viroplasms characteristically present during the early stages of infection are competent in driving high levels of dsRNA. Instead of being associated with genome replication, the increase of viroplasm size during the viral life cycle seems more likely to be intertwined with the high degree of DLP assembly and DLP-to-TLP conversion that occur at the later stages of infection. In fact, our data raise the possibility that viroplasms transition from an early-stage function of promoting genome packaging and replication to a late-stage function of promoting DLP assembly and egress. It is possible that the transition in function is mediated by the NSP4-dependent recruitment of transcriptionally active DLPs from the viroplasm, which could have the effect of down-regulating genome packaging and replication by removing sources of plus-strand RNA templates.

NSP4 loss of function was also correlated with the accumulation of significant levels of empty particles, particularly DLPs, in the infected cell. This may be a consequence of the failure of structural proteins to appropriately localize to viroplasms in NSP4 siRNA-transfected cells, where the assembly of core and DLPs seem more likely to be coordinated with genome packaging and replication. With the increased levels of
VP2 and VP6 accumulating outside of viroplasms in the NSP4 siRNA-transfected cells, the intrinsic capacities of the inner capsid proteins to self assemble with one another could be expected to generate empty particles. Irrespective of the origin of the empty particles, their accumulation suggests an important role for NSP4 in linking the processes of capsid morphogenesis and genome packaging.

Our results extend the recently published findings of Lopez et al. (15), who also showed that NSP4 loss of function resulted in a decrease in the assembly of packaged particles, a failure of maturation of viroplasms, and the maldistribution of nonstructural and structural proteins in the cytoplast. The results of these studies support a conclusion that NSP4 is an unusually multifaceted protein whose functions serve to link the earliest events of the viral life cycle with those later events that culminate in final capsid assembly. The fact that NSP4 expression was successfully knocked down by a g10-specific siRNA without affecting g10 replication reaffirms the belief that two pools of plus-strand RNAs are present in the infected cell, one contained in viroplasms, which is resistant to degradation by RISCs, and another contained in the cytosol, which is susceptible to degradation by such complexes (6, 26). The location of the transcriptionally active DLPs, relative to these two pools, that give rise to the elevated levels of plus-strand RNAs in infected cells containing NSP4 siRNAs remains to be determined.

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