Reduced expression of the rotavirus NSP5 gene has a pleiotropic effect on virus replication

Tomás López, Margarito Rojas, Camilo Ayala-Bretón, Susana López and Carlos F. Arias

Correspondence
Carlos F. Arias
arias@ibt.unam.mx

Departamento de Genética del Desarrollo y Fisiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Avenida Universidad 2001, Col. Chamilpa, Cuernavaca, Morelos 62210, Mexico

Received 15 December 2004
Accepted 20 March 2005

Rotavirus RRV gene 11 encodes two non-structural proteins, NSP5 and NSP6. NSP5 is a phosphorylated non-structural protein that binds single- and double-stranded RNA in a non-specific manner. Transient expression of this protein in uninfected cells has provided evidence for its participation in the formation of electron-dense cytoplasmic structures, known as viroplasms, which are thought to be key structures for the replication of the virus. NSP6 is a protein of unknown function that seems not to be essential for virus replication in cell culture. To study the function of NSP5 in the context of a viral infection, the expression of RRV gene 11 was silenced by RNA interference. Reduction in the synthesis of NSP5, as shown by immunoblot and immunofluorescence assays, correlated with a reduction in the number and size of viroplasms and with an altered intracellular distribution of other viroplasm-associated proteins. Silencing of gene 11 also resulted in a reduced synthesis of viral RNA(+) and double-stranded RNA and of all viral proteins, as well as in a decreased production of infectious virus. A similar phenotype was observed when the NSP5 coding gene of the lapine rotavirus strain Alabama was silenced. The fact that the NSP5 gene of rotavirus Alabama lacks the AUG initiator codon for a complete NSP6 protein, suggests that the described phenotype in gene 11-silenced cells is mostly due to the absence of NSP5. The data presented in this work suggest that NSP5 is a key protein during the replication cycle of rotaviruses.

INTRODUCTION

Rotaviruses, members of the family Reoviridae, have a genome composed of 11 segments of double-stranded RNA (dsRNA) that encodes six structural (VP1 to VP4, VP6 and VP7) and six non-structural (NSP1 to NSP6) proteins. The genome is enclosed in a capsid formed by three concentric layers of protein. The innermost layer, formed by VP2, contains the viral genome, the viral RNA polymerase VP1 and the capping enzyme VP3, which constitute the core of the virus. The addition of VP6 outside the VP2 layer produces double-layered particles (DLPs). The outermost layer, characteristic of triple-layered infectious particles (TLPs), is composed of two proteins, VP4 and VP7. The smooth external surface of the virus is formed by glycoprotein VP7, while 60 spike-like structures, formed by VP4, extend from the VP7 surface (Estes, 2001).

During the process of infection, the infecting TLP is adsorbed, penetrates the plasma membrane and is uncoated, loosing the two outer layer proteins and yielding a transcriptionally active DLP. The viral polymerase VP1 then synthesizes the primary viral transcripts, which in addition to direct the synthesis of viral proteins, i.e. to function as mRNAs, are also believed to serve as RNA templates [RNA(+)] for the synthesis of the RNA-negative strands [RNA(−)] to form the genomic dsRNA (Estes, 2001; Patton et al., 2003). Once a critical mass of viral proteins is synthesized, viral polypeptides NSP2, NSP5, NSP6, VP1, VP2, VP3 and VP6 accumulate in electron-dense cytoplasmic inclusions known as viroplasms (González et al., 2000; Mattion et al., 1991; Petrie et al., 1984); these are key structures in the replication of rotavirus, where the synthesis of dsRNA and assembly of progeny DLPs are thought to take place (Estes, 2001).

The synthesis of RNA(−) has been proposed to occur concurrently with the packaging of RNA(+) into core replication intermediate (RI) particles (formed by VP1, VP2, VP3, and the non-structural proteins NSP2 and NSP5) in a highly coordinated manner, such that packaging and replication of RNA(+) lead to the formation of cores containing one copy of each of the 11 dsRNA genome segments (Patton et al., 2003). Assembly of VP6 onto core RIs is then believed to lead to the production of transcriptionally active, dsRNA-containing DLPs (Estes, 2001). These particles are thought...
to initiate a second round of transcription, which results in an amplified second wave of viral protein synthesis and assembly of DLPs, as has been shown for reovirus (Nibert & Schiff, 2001). Finally, the assembled DLPs bud through the membrane of the ER, acquiring during this process a transient membrane envelope, which is subsequently removed to yield mature infectious TLPs (Estes, 2001; Patton, 1995). Although the mechanism through which the transient membrane envelope is lost is not known, it has been recently shown to depend on VP7 (López et al., 2005).

Rotavirus gene 11 encodes two proteins, NSP5 and NSP6. NSP5 is an O-glycosylated phosphoprotein (Afrikanova et al., 1996; González & Burrone, 1991) present in several phosphorylated isoforms, which are thought to regulate their own phosphorylation (Afrikanova et al., 1996; Blackhall et al., 1998; Eichwald et al., 2004; Eichwald et al., 2002). NSP5 has been shown to interact in a sequence independent fashion with dsRNA and single-stranded (ss) RNA (Vende et al., 2002), and it has been suggested that its phosphorylation state influences the rate of translation versus replication of the viral RNA (Chnaiderman et al., 2002). When co-expressed with NSP2, in the absence of other viral proteins, NSP5 forms viroplasm-like structures (Fabbretti et al., 1999), and more recently it was shown that amino-terminal deletion mutants of this protein can form viroplasm-like structures in the absence of NSP2 (Mohan et al., 2003). NSP5 has also been shown to have a strong affinity to VP2, to an extent that it can outcompete VP6 in VLPs (Berois et al., 2003). Despite the extensive characterization of the biochemical properties of NSP5, and the description of its viroplasm-forming properties by transient expression in uninfected cells, the role and relevance of this protein in the context of virus replication has not been determined.

NSP6 is a 92 aa protein of unknown function, encoded in a +1 alternative open reading frame (ORF) of rotavirus gene 11 (Matton et al., 1991). The NSP5 gene of many rotavirus strains has an ORF that could potentially encode NSP6; however, its synthesis has only been demonstrated in a few rotavirus strains (Matton et al., 1991; Torres-Vega et al., 2000). In addition, the fact that some rotaviruses do not have this ORF suggests that NSP6 is not essential for virus replication (Matton et al., 1991; Taraporewala & Patton 2004; Torres-Vega et al., 2000).

RNA interference (RNAi) is a process triggered by dsRNA that specifically silences the expression of the gene which shares sequence identity with the triggering dsRNA. The recent implementation of the RNAi technology to efficiently silence gene expression in mammalian cells has opened a tremendous opportunity for a rapid advance in the characterization of gene function (Elbashir et al., 2001). This technology has also proven to be extremely useful to study the function of genes from plant and animal viruses, including rotaviruses (Arias et al., 2004; Doctor et al., 2002; López et al., 2005; Silvestri et al., 2004). To evaluate directly the role of NSP5 in the context of the replication of rotaviruses in infected cells, we silenced the expression of rotavirus RRV gene 11 by RNAi. We found that reduced amounts of NSP5 correlated with the inhibition of genomic dsRNA and viral protein synthesis, as well as with a marked reduction in the production of infectious viral progeny. These data indicate an essential role of NSP5 in the replication of rotaviruses, and give further insight into the function this protein plays in the virus replication cycle.

**METHODS**

**Cells, viruses and antibodies.** The monkey kidney epithelial cell line MA104 was grown in Eagle’s minimal essential medium (MEM) supplemented with 10% fetal bovine serum, and was used for all experiments carried out in this work. The reovirus strain RRV (PSB,G3) and the human rotavirus strain Wa (P1A,G1) were obtained from Dr. H. B. Greenberg, Stanford University, Stanford, CA; the bovine rotavirus strain RF (P6,G6) was kindly provided by Dr. J. Cohen, CNRS-INRA, Gif-sur-Yvette, France; and the lapine strain Alabama (P14,G3) was obtained from Dr. M. Conner, Baylor College of Medicine Houston, TX. All rotavirus strains were propagated in MA104 cells as described previously (Pando et al., 2002). Monoclonal antibodies (mAbs) to VP2 (mAb 3A8), VP6 (mAb 255/60), NSP4 (mAb B4) and VP7 (mAb 60) were kindly provided by Dr. H. B. Greenberg. Rabbit polyclonal sera to NSP2 and NSP5 have been described previously (González et al., 2000). Polyclonal antibodies to purified RRV TLPs were produced in rabbits as described previously (López et al., 2005). Antibodies to vimentin were raised in rabbits by immunization with human recombimant vimentin produced in bacteria, expressed from a plasmid kindly provided by Dr. J. F. Hess, University of California, Davis, CA. Polyclonal antibodies to a fragment of RRV VP4 (aa 474–776) synthesized in, and purified from, bacteria as described previously (Zarate et al., 2003), were produced in rabbits. Alexa-488- and -568-conjugated secondary antibodies were purchased from Molecular Probes (Eugene). Horseradish peroxidase-conjugated goat anti-rabbit polyclonal antibody was from Perkin Elmer Life Sciences and horseradish peroxidase-conjugated rabbit anti-mouse IgG was from Zymed.

**Sequence and lipofection of small interfering RNAs (siRNAs).** For this work two NSP5 siRNAs corresponding to nt 95–115 (siRNA<sup>NSP5-1</sup>) and 244–264 (siRNA<sup>NSP5-2</sup>) of rotavirus RRV gene 11 (Mohan & Atreya, 2001) were purchased from Dharmacon Research. siRNA<sup>NSP5-1</sup> had the sequence 5'–GUUCACCUCCUUCAACUAGATT-3' (antisense), 5'–GUUCACCCAUCAACUAUGATT-3' (sense), while siRNA<sup>NSP5-2</sup> had the sequence 5'–GACAAAGCAGCGCCUGGCTCT-3' (sense), 5'–GGCAGCGCCAGUUCUGGCTT-3' (antisense). Both NSP5 siRNAs silenced the expression of gene 11 with similar efficiency. As a negative control a previously reported lamin siRNA was used (Dector et al., 2002; Elbashir et al., 2001). Confluent monolayers of MA104 cells in 48- or 96-well plates were lipofected with 100 or 50 µl, respectively, of a mixture containing 40 µg lipofectamine (Invitrogen) ml<sup>−1</sup> and 600 pmol siRNAs ml<sup>−1</sup> in MEM without serum. The transfection mixture was added to cells previously washed with MEM and incubated for 8 h at 37°C. After this time the transfection mixture was removed, the cells were washed with MEM and kept in this medium without serum or antibiotics for 36 h at 37°C, before being infected at an m.o.i. of 3.

**Infection of cells and titration of viral progeny.** Cell monolayers in 96-well plates were infected with an m.o.i. of 3 as described
RESULTS

Silencing the expression of rotavirus gene 11 reduces viral progeny and viral protein synthesis

To study the relevance of gene 11 products in the context of a viral infection, two siRNAs (siRNA<sup>NSP5-1</sup> and siRNA<sup>NSP5-2</sup>) corresponding to the sequence of rotavirus RRV gene 11 (Mohan & Atreya, 2001) were synthesized and transfected into MA104 cells. The cell monolayers were infected 36 h post-transfection with rotavirus strain RRV at an m.o.i. of 3, and 14–16 h later the titre of viral progeny produced was determined by a focus-forming assay. The two siRNAs tested interfered with the replication of RRV with similar efficiency; the infectious viral progeny produced in cells transfected with either siRNA was reduced by 70–80 % as compared with the yield of RRV obtained from cells transfected with an irrelevant, control siRNA (Table 1). To confirm that the synthesis of NSP5 was being specifically blocked by the siRNAs, cells transfected with siRNA<sup>NSP5-1</sup> were infected with rotavirus RRV, and 12 h p.i. the viral proteins were analysed by immunoblotting, using a mixture of rabbit polyclonal sera to NSP5, to purified RRV TLPs, and to vimentin as an internal loading control (Fig. 1a).

As expected, the amount of NSP5 was drastically reduced, however, a reduction in the level of the structural viral proteins VP4, VP6 and VP7 was also observed, while the level of vimentin was not altered. The general reduction in the synthesis of both structural and non-structural viral proteins was confirmed by SDS-PAGE analysis of viral proteins metabolically labelled with an 35S-labelling mix, produced in an infection made in the presence or absence of the siRNA to NSP5 (Fig. 1b).

It has been reported that siRNAs can activate the interferon system, causing a non-specific inhibition of cell protein synthesis.

![Table 1. Effect of silencing gene 11 on rotavirus progeny yield (%)](http://vir.sgmjournals.org/)

<table>
<thead>
<tr>
<th>siRNA</th>
<th>RRV</th>
<th>Rotavirus strain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RF</td>
<td>Wa</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>NSP5-1</td>
<td>28 ± 4.5</td>
<td>32 ± 6.3</td>
</tr>
<tr>
<td>NSP5-2</td>
<td>20 ± 6.4</td>
<td>ND</td>
</tr>
</tbody>
</table>

*The viral progeny titre in siRNA control-transfected cells was 2 × 10<sup>6</sup> focus-forming units ml<sup>−1</sup> for RRV, 1 × 10<sup>5</sup> for RF, 5 × 10<sup>5</sup> for Wa, 6 × 10<sup>5</sup> for Alabama. The arithmetic mean and standard deviation of four independent experiments performed in duplicate are shown.
synthesis (Kim et al., 2004; Persengiev et al., 2004; Sledz et al., 2003). To evaluate if the general inhibition of the synthesis of viral protein induced by siRNA NSP5-1 was specific, we analysed the effect of this siRNA on the production of viral progeny and viral protein synthesis of the human rotavirus strain Wa and the bovine rotavirus strain RF. The sequence of siRNA \( \text{NSP5}^{-1} \) corresponding to RRV gene 11 is identical in rotavirus RF, but differs in rotavirus Wa gene 11 by 2 nt (at positions 14 and 15 of the sense sequence). If the siRNA is inducing a non-specific effect, the production of the viral progeny of all three viruses should be affected. As shown in Table 1, siRNA \( \text{NSP5}^{-1} \) did not affect the production of rotavirus Wa progeny, but had a strong effect in the case of RF, similar to that observed for RRV. Also, immunoblot analysis of the viral proteins synthesized in the presence of siRNA \( \text{NSP5}^{-1} \) showed that this siRNA, but not a control siRNA, reduced the accumulation of NSP5 and other viral proteins in cells infected with rotavirus RF, but not in cells infected with rotavirus strain Wa (Fig. 1c). These results indicate that the reduction in viral progeny and viral protein expression is a consequence of the specific inhibition of the expression of gene 11 induced by siRNA \( \text{NSP5}^{-1} \).

**Interference of rotavirus gene 11 expression reduces the general synthesis of viral mRNAs and genomic dsRNA**

To analyse the effect of silencing the expression of gene 11 on the synthesis of viral messenger and genomic RNA, siRNA \( \text{NSP5}^{-1} \)-transfected cells were infected with RRV, the cells were labelled with \( ^{32}\text{P} \)orthophosphate for 10 h, starting 2 h p.i., followed by the extraction of total RNA from the cell lysates and analysis by PAGE, using the conditions described by Silvestri et al. (2004) that allow the detection of viral mRNAs and most segments of dsRNA (Fig. 2a). A reduction in the accumulation of all viral transcripts and dsRNA was observed in the presence of siRNA \( \text{NSP5}^{-1} \) as compared with cells transfected with an irrelevant siRNA. The amount of viral dsRNA synthesized was also analysed by PAGE and silver staining of unlabelled dsRNA extracted at 12 h p.i. Similar to what was observed for \( ^{32}\text{P} \)-labelled RNA, the amount of dsRNA was also found to be diminished in cells transfected with siRNA \( \text{NSP5}^{-1} \), as compared with cells transfected with a control siRNA or with an siRNA to RRV VP4 (Dector et al., 2002) (Fig. 2b).

**Interfering with the expression of NSP5 in a rotavirus strain lacking a complete ORF for NSP6**

Gene 11 of some rotavirus strains does not contain the complete NSP6 ORF present in most strains, which potentially encodes a protein of 92 aa. In some cases, e.g. rotavirus strain OSU, the NSP6 ORF encodes a 51 aa protein, while in others, like rotavirus strains Alabama or Mc323, there is no initiator AUG at the beginning of the potential second ORF, but it is not known whether a shorter NSP6, starting from a downstream AUG could be synthesized (Fig. 3b). To test if NSP6 contributes to the phenotype observed when gene 11 is silenced, we characterized the yield of viral progeny and viral protein synthesis in cells infected with the lapine rotavirus strain Alabama in the presence of an NSP5 siRNA. Rotavirus strain Alabama was chosen, since rotavirus OSU has already been tested (Campagna et al., 2003), and rotavirus strain Mc323 was not available. Since
siRNA_NSP5-1 has two mismatches with the target sequence of Alabama gene 11, while we used siRNA_NSP5-2, whose target sequence is identical in rotaviruses RRV and Alabama. The interference of gene 11 expression in Alabama reduced the production of viral progeny (Table 1) and the synthesis of viral protein (Fig. 3a), although the inhibition of viral progeny was somewhat lower (50%) as compared with that achieved with RRV (80%).

**Intracellular distribution of several viral proteins is altered in gene 11-silenced cells**

The level of reduction achieved in the titre of RRV viral progeny and viral protein synthesis in cells transfected with siRNA_NSP5-1 is consistent with the efficiency of transfection achieved with this siRNA, of about 70% (data not shown and Dector et al., 2002). These results suggest that the viral proteins and infectious virus produced in the cells successfully transfected with siRNA_NSP5-1 is none or very low. Thus, to determine the effect of silencing rotavirus gene 11 on the synthesis of NSP5 and other viral proteins at the individual cell level, siRNA_NSP5-1-transfected cells were infected with RRV at an m.o.i. of 3, and examined by immunofluorescence with mono-specific sera to different proteins. NSP5 was still detectable and localized to viroplasms in most cells, although the number and size of viroplasms was reduced as compared with cells transfected with an siRNA control (Ctrl), siRNA_NSP5-1 (NSP5) or an siRNA to rotavirus RRV VP4 gene (VP4). The extracted dsRNA segments were separated by PAGE and detected by silver staining. The position of the genes 4 and 11 encoding VP4 and NSP5, respectively, is indicated. The same volume fraction from the total volume collected for each sample was loaded in the gel.

**Fig. 2.** Silencing rotavirus gene 11 decreases the synthesis of viral RNA. (a) Total RNA was purified from RRV-infected cells metabolically labelled with [32P]orthophosphate that had been previously transfected with either an siRNA control (Ctrl) or siRNA_NSP5-1 (NSP5). The viral mRNAs and dsRNA genes were separated by PAGE under conditions as described in Methods and detected by a PhosphorImager. The genomic dsRNA segments (indicated at left) and the viral mRNAs (indicated at right) were identified by running 32P-labelled viral dsRNA as a marker (not shown). (b) Total dsRNA was purified from cell lysates of RRV-infected cells that had been previously transfected with either an siRNA control (Ctrl), siRNA_NSP5-1 (NSP5) or an siRNA to rotavirus RRV VP4 gene (VP4). The extracted dsRNA segments were separated by PAGE and detected by silver staining. The position of the genes 4 and 11 encoding VP4 and NSP5, respectively, is indicated. The same volume fraction from the total volume collected for each sample was loaded in the gel.

**Fig. 3.** Silencing the NSP6 gene of rotavirus Alabama reduces the general synthesis of viral proteins. (a) Immunoblot analysis of rotavirus structural proteins (strains RRV and Alabama –Ala – as indicated) synthesized in cells transfected with either siRNA_NSP5-2 (NSP5) or an irrelevant siRNA (Ctrl), as indicated. Vimentin was used as an internal loading control. The transferred proteins were incubated with a mixture of polyclonal antibodies to purified rotavirus RRV, NSP5 and vimentin, and the bound antibodies were developed by incubation with a peroxidase-labelled anti-rabbit immunoglobulin antibody. (b) Schematic representation of RRV NSP5 and the NSP6 protein from rotavirus strains RRV, Alabama and OSU. NSP6 and NSP5 are placed according to their relative positions in gene 11 of the ORFs that encode them. Alabama NSP6 is depicted with a dotted line, since it is not known if this protein is synthesized in infected cells.
of this protein with microtubules (González et al., 2000; Nejmeddine et al., 2000) was maintained and detected in both control and NSP5-silenced cells (see arrows in Fig. 4, panel VP4). Similar to VP4, the distribution of NSP4 also changed from a viroplasm-associated ring-like appearance (González et al., 2000) to a more dispersed finely punctated pattern. VP7 also changed its distribution from a predominant ring-like pattern associated with viroplasms (González et al., 2000) in control cells, to a more diffuse perinuclear signal. The more drastic change observed, however, was the distribution of VP6, where most of the protein shifted from a viroplasm location to a fibrous array in the cytoplasm. The fibres of VP6 did not co-localize with tubulin, actin or vimentin (data not shown).

**Silencing the expression of gene 11 blocks late viral transcription and protein synthesis**

The reduced synthesis of viral protein, mRNA, dsRNA and progeny particles described above, suggests that the inhibition of gene 11 expression blocks the replication of the viral genome and the putative second round of transcription. If this is correct, one would expect that the primary synthesis of viral mRNA and protein that takes place early after infection would not be affected (except for NSP5), but the inhibition would be evident rather late in the replication cycle, when the secondary round of transcription and translation occurs. To test this hypothesis, MA104 cells were infected with rotavirus RRV at an m.o.i. of 3, and the infected cells were pulse-labelled every hour for 30 min with a35S-labelling mix, starting 1 h after infection (Fig. 5a). The level of NSP5 was detected by immunoblot using the same samples shown in Fig. 5(a) (Fig. 5b). The inhibition of the synthesis of NSP5 was already evident at 2 h p.i., a time point at which this protein was first detected in control cells. In contrast, the synthesis of the rest of the viral proteins was similar in the NSP5 siRNA- and control siRNA-transfected cells until 3 h p.i., while the inhibition of their synthesis started to be noticed at 4 h p.i. and was maximal at 6–8 h p.i.

We also determined the accumulation of gene 10 RNA(+).
Rotavirus NSP5 is essential for virus replication

DISCUSSION

It has been suggested that rotavirus non-structural protein NSP5 is involved at several stages during the replication of the viral genome, including the formation of viroplasms, the transport of viral RNAs (+) to these inclusions, and the preparation of these RNAs for packaging and replication (Patton et al., 2003). NSP5 has also been implicated in the regulation of the translation of viral mRNAs (Chnaiderman et al., 2002) and, given its capacity for binding dsRNA, in blocking the interferon-induced RNA-dependent activation of protein kinase PKR (Vende et al., 2002). However, with exception of its ability to form viroplasm-like structures when transiently expressed in uninfected cells (Fabbretti et al., 1999; Mohan et al., 2003), there is no direct evidence either in vivo or in vitro for its suggested functions. In this work, we studied the role of NSP5 in the context of a viral infection, using RNAi technology to suppress gene expression in mammalian cells.

Although the RNAi response induced by siRNA$^{\text{NSP5-1}}$ most probably inhibits the synthesis of both NSP5 and NSP6, the block in virus replication, as well as the reduced synthesis of viral components observed in this work is most likely the result of the impaired levels of synthesis of NSP5. This conclusion is supported by the following observations: (i) an ORF encoding a full-length NSP6 is not present in gene 11 of some group A rotaviruses (strains Mc323, Alabama, 512-C and OSU – GenBank accession numbers U54772, J04361, AB008662 and X15519, respectively) and is missing in all group C rotavirus strains (Torres-Vega et al., 2000), suggesting that NSP6 is not essential for the replication of the virus; (ii) the expression of intracellular antibodies to NSP5 caused a phenotype similar to that described in this work (Vascotto et al., 2004); (iii) silencing the expression of gene 11 of porcine rotavirus strain OSU, which encodes a truncated NSP6 ORF, resulted in the general reduction of viral protein synthesis and inhibition of rotavirus replication (Campagna et al., 2003); and (iv) the inhibition effect of siRNA$^{\text{NSP5-1}}$ on the replication and protein synthesis of rotavirus Alabama described in this work was similar to that observed for rotavirus RRV. The gene 11 of Alabama strain does not have an AUG codon at the beginning of the ORF that would code for a 92 aa NSP6 protein, like most other rotavirus strains. It rather has an in-phase downstream AUG that could potentially code for a protein of 79 aa. If this protein is indeed synthesized and if it is functional, it would imply that the 79 carboxy-terminal amino acids of NSP6 are enough to sustain its function. Furthermore, as mentioned above, rotavirus OSU gene 11 produces a 51 aa truncated version of NSP6, and its silencing also causes a reduction of viral protein and genomic dsRNA synthesis (Campagna et al., 2003). Although it is not possible to discard a central role of NSP6 in rotavirus replication, these observations suggest that most probably NSP6 plays a regulatory role of the NSP5 activity, as previously suggested (Torres-Vega et al., 2000), a role that might have been substituted somehow in the strains that lack the full-length NSP6.

![Image](https://vir.sgmjournals.org/article-figures/1615/5.jpg)

Fig. 5. Reduction in NSP5 expression causes a time-dependent decrease of viral RNA and protein synthesis. (a) MA104 cells lipofected with either an irrelevant siRNA (C) or siRNA$^{\text{NSP5-1}}$ (N) were infected with rotavirus RRV, and at indicated times p.i. the cells were pulsed-labelled for 30 min with an $^{35}$S-labelled mix. The cells were then lysed in Laemmli sample buffer and the proteins separated by SDS-PAGE and detected by autoradiography. (b) The same samples described in (a) were transferred onto a nitrocellulose membrane and the accumulated NSP5 was analysed by immunoblot using an antibody to NSP5. The amount of protein loaded in each lane was previously adjusted by eye inspection of Coomassie blue-stained gels. (c) Cells lipofected with either control (open bars) or NSP5-1 (closed bars) siRNAs, as indicated, were infected with rotavirus RRV, and at the indicated times (T0, T3, T6, and T9 at 0, 3, 6 and 9 h p.i., respectively) total RNA was extracted with Trizol, and the levels of gene 10 RNA were determined by real-time RT-PCR. The results are expressed as fold-increase over the levels detected at time zero of infection (immediately after the end of the virus adsorption period). The arithmetic mean, with one standard deviation, of two independent experiments is shown.

at different times p.i. by semi-quantitative RT-PCR (Fig. 5c). Gene 10 was selected as marker of the general synthesis of viral RNA, since the RT-PCR conditions for its measurement were already set up in the laboratory (C. Ayala-Breton and others, unpublished data). Similar to what was observed for protein synthesis, at 3 h p.i. the gene 10 RNA (+) levels were similar in cells infected in the presence of the siRNA$^{\text{NSP5-1}}$, as compared to control cells, while a clear difference between these two conditions was found at 6 h p.i.
Gene 11-silenced cells showed fewer and smaller viroplasms than cells transfected with the control siRNA, as well as a partial delocalization of viroplasmic proteins VP2, NSP2 and VP6 (Fig. 4), strongly suggesting that NSP5 is essential to nucleate the formation of these cytoplasmic inclusions (Fabbretti et al., 1999). It would appear that in the absence of a regular amount of NSP5 the number and size of viroplasms decreases, causing that only a fraction of viroplasmic proteins anchors to these structures, while the ‘excess’ of these proteins redistributes in the cytoplasm. In contrast to NSP2 and VP2, whose ‘excess’ became homogeneously distributed in the cytoplasm, VP6 showed a dramatic redistribution into fibrous structures that appear to extend to the periphery of the cell. The reason for this fibrous pattern of VP6 is not clear, since the VP6 fibres did not co-localize with vimentin, actin or tubulin, and had variable thickness (data not shown). The distribution of VP6 into fibres was also observed when the expression of NSP4 was silenced (López et al., 2005); in that case, the amounts of NSP2 and NSP5 were also reduced, and the mean size of the viroplasms was also smaller, suggesting that the distribution of VP6 is sensitive to the concentration of proteins that accumulate in viroplasms and that treatments that alter this concentration cause VP6 to redistribute as fibres. The reason for this delocalization is not clear, but NSP5 might have an unrecognized role in the recruitment of viral proteins to viroplasms. Direct interaction with NSP5 has been demonstrated for NSP6, NSP2, VP1 and VP2, and changes in the distribution of VP2 were observed when NSP5 was co-expressed in insect cells (Berois et al., 2003). In the case of VP6, there are no reports describing a direct interaction with NSP5 and, in fact, NSP5 has been reported to dislodge VP6 from virus-like particles formed by VP2 and VP6 (Berois et al., 2003). Recently, it was shown that the inhibition of NSP2 expression also inhibits viroplasm formation, genome replication, virus assembly, and the synthesis of the viral proteins (Silvestri et al., 2004); however, the intracellular distribution of the viral proteins in NSP2-deficient cells was not characterized. VP4, VP7 and NSP4, which are not integral viroplasmic proteins but are closely associated to these structures (González et al., 2000), were also delocalized in gene 11-silenced cells, probably as an indirect effect of the reduced size and number of viroplasms.

The data presented in this work support the existence of a second round of transcription driven by de novo synthesized DLPs (Fig. 5). The impaired number and size of viroplasms produced in gene 11-silenced cells probably causes a decreased production of DLPs that results in a deficient secondary synthesis of viral mRNA, with the consequent reduction of: (i) the synthesis of viral proteins and genomic dsRNA; (ii) the formation of ‘healthy’ viroplasms; and (iii) the assembly of DLPs, and eventually infectious TLPs. As predicted by this model, early after virus infection (3 h or earlier) of siRNA<sub>NSP5</sub>-transfected cells only the accumulation of NSP5 (the siRNA target) is clearly affected (Fig. 5a), while the synthesis of other viral proteins and of RNA(+), as judged from the levels of gene 10 RNA, are not affected, indicating that at the beginning of infection the RNA(+) is mostly produced by the parental infecting DLP. At later times (4 h p.i. and later) all viral proteins and viral RNA(+) start to be inefficiently produced because, in the absence of NSP5, viroplasms are scarce, and a limited amount of progeny DLPs are assembled, with the consequent reduction in viral protein and RNA(+), and an inhibition of the production of progeny infectious virus. Experiments are currently being carried out in our laboratory to characterize in detail the timing of transcription and replication of the virus genome during the rotavirus infection cycle, and the effect on this timing of silencing the expression of various structural and non-structural viral proteins.

ACKNOWLEDGEMENTS

We are grateful to Andrés Saralegui for his excellent technical assistance with the confocal microscope and to Pedro Romero for preparing and purifying the viruses. This work was partially supported by grants 55003662 and 55000613 from the Howard Hughes Medical Institute, and G37621N from the National Council for Science and Technology-Mexico.

REFERENCES


Rotavirus NSP5 is essential for virus replication


