Rotavirus variant replicates efficiently although encoding an aberrant NSP3 that fails to induce nuclear localization of poly(A)-binding protein

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The rotavirus (RV) non-structural protein NSP3 forms a dimer that has binding domains for the translation initiation factor elf4G and for a conserved 3'-terminal sequence of viral mRNAs. Through these activities, NSP3 has been proposed to promote viral mRNA translation by directing circularization of viral polysomes. In addition, by disrupting interactions between elf4G and the poly(A)-binding protein (PABP), NSP3 has been suggested to inhibit translation of host polyadenylated mRNAs and to stimulate relocalization of PABP from the cytoplasm to the nucleus. Herein, we report the isolation and characterization of SA11-4Fg7re, an SA11-4F RV derivative that contains a large sequence duplication initiating within the genome segment (gene 7) encoding NSP3. Our analysis showed that mutant NSP3 (NSP3m) encoded by SA11-4Fg7re is almost twice the size of the wild-type protein and retains the capacity to dimerize. However, in comparison to wild-type NSP3, NSP3m has a decreased capacity to interact with elf4G and to suppress the translation of polyadenylated mRNAs. In addition, NSP3m fails to induce the nuclear accumulation of PABP in infected cells. Despite the defective activities of NSP3m, the levels of viral protein and progeny virus produced in SA11-4Fg7re- and SA11-4F-infected cells were indistinguishable. Collectively, these data are consistent with a role for NSP3 in suppressing host protein synthesis through antagonism of PABP activity, but also suggest that NSP3 functions may have little or no impact on the efficiency of virus replication in widely used RV-permissive cell lines.

INTRODUCTION

Viruses often bring about changes to the cellular translation machinery to favour production of viral proteins necessary for the viral life cycle (Dreher & Miller, 2006; Powell, 2010). Translation initiates with the assembly of the elf4F cap-binding complex, consisting of the cap-binding protein elf4E, the RNA helicase elf4A and the scaffolding protein elf4G (Groppo & Richter, 2009). The elf4F complex binds to the 5’ end of mRNAs via the cap-binding protein elf4E, which is also bound to the elf4G scaffold. Simultaneously, elf4G interacts with the poly(A)-binding protein (PABP), which binds to the 3’ poly(A) tail of mRNAs (Magnus et al., 2003). These interactions are thought to stimulate translation by circularizing mRNAs (Amrani et al., 2008; Kahvejian et al., 2001). The 40S ribosomal subunit is then recruited to mRNAs via elf4G binding to the ribosome-associated initiation factor elf3 (Gingras et al., 1999). The 40S subunit scans downstream along the mRNA until recognizing an appropriate initiation codon. The large 60S ribosomal subunit then interacts with the 40S subunit forming the complex that directs protein synthesis (Fromont-Racine et al., 2003).

Rotavirus (RV) contains a genome consisting of 11 segments of dsRNA (Estes & Kapikian, 2007). Transcription of the genome produces 11 species of viral mRNAs that contain 5’ cap structures but lack 3’ poly(A) tails (Imai et al., 1983; Lawton et al., 2000). Instead, nearly all RV mRNAs end with the 3’-consensus sequence (3’CS) 5’-UGUGACC-3’ (Mitchell & Both, 1990). The RV 36 kDa non-structural protein NSP3 assembles into a stable homodimer that contains structurally independent functional domains (Piron et al., 1999). In the dimer, the N-terminal regions of the two NSP3 monomers intertwine to form an asymmetrical RNA-binding pocket that recognizes the GACC portion of the 3’CS (Deo et al., 2002; Poncet et al., 1993, 1994). At the other end of the dimer, the C-terminal regions of the monomers interact to form a symmetrical elongated structure that contains two binding pockets for elf4G (Groft & Burley, 2002; Piron et al., 1998). Through its co-affinity for GACC and elf4G, the NSP3 dimer is predicted to promote viral mRNA circularization (Groft & Burley, 2002; Vende et al., 2000).
The capacity of NSP3 to out-compete PABP for eIF4G is believed to inhibit the formation of eIF4G–PABP complexes needed for efficient translation of polyadenylated cellular mRNAs (Groft & Burley, 2002; Piron et al., 1998). It is through this action that NSP3 is presumed to mediate shutdown of host protein synthesis. Indeed, expression of recombinant NSP3 in uninfected BSC1 cells has been shown to suppress the translation of polyadenylated cellular RNAs (Padilla-Noriega et al., 2002). Moreover, knockdown of NSP3 expression in RV-infected cells by NSP3-specific siRNAs (small interfering RNAs) restores the efficient translation of polyadenylated mRNAs (Montero et al., 2006). Although PABP typically accumulates in the cytoplasm, the protein will shuttle to the nucleus where it engages and subsequently chaperones host transcripts to the cytoplasm (Afonina et al., 1998). Unlike uninfected cells, PABP accumulates in the nucleus of RV-infected cells. Based on transient expression assays, NSP3 is responsible for this phenomenon (Harb et al., 2008; Montero et al., 2008).

Surprisingly, despite the crucial roles proposed for NSP3 in the RV life cycle, RNAi (RNA interference) experiments have indicated that knockdown of NSP3 expression has no effect on viral protein synthesis in infected cells. Rather, NSP3 knockdown was linked to higher levels of virus progeny formation (Montero et al., 2006). Thus, the role of NSP3 in the RV life cycle remains uncertain. Although partial success has been achieved in establishing a reverse genetics system for generating NSP3-mutated RVs, the present system is not sufficiently tractable for routine use in NSP3 function studies (Troupin et al., 2010). As a result, the isolation of naturally occurring RV mutants expressing altered NSP3 remains an important alternative for examining the protein’s function in the context of infection.

Here, we describe the characteristics of an RV variant (SA11-4Fg7re) that contains a sequence rearrangement initiating within its NSP3 ORF. Despite producing a mutant NSP3 (NSP3m) nearly twice the size of wild-type NSP3, we found that the variant virus grew just as well as its wild-type counterpart in vitro. However, unlike the wild-type virus, the variant did not induce nuclear accumulation of PABP, and its NSP3m product showed a decreased capacity to interact with eIF4G or to suppress translation of polyadenylated mRNA. While our findings support the idea that NSP3 has an important role in the shutdown of host protein expression, our findings also suggest that some or all of the activities prescribed for NSP3 may be non-essential in the highly permissive cell lines commonly used for the study of the RV life cycle.

**RESULTS**

**SA11-4F variant with an atypical gene 7 RNA**

RV strain SA11-4F was serially passaged into MA104 cells, using low dilutions of infected cell lysates as inoculum. Viral dsRNAs in the lysates were examined by PAGE at each passage to monitor for the emergence of variant viruses with novel genome constellations. A variant with an atypical genome (SA11-4Fg7re) in the passage 24 lysate was isolated by plaque purification. The genome of SA11-4Fg7re lacked the wild-type gene 7 dsRNA, but instead contained a large dsRNA migrating slightly slower than the gene 5 dsRNA (Fig. 1a). Analysis of single-step growth curves showed that wild-type SA11-4F and SA11-4Fg7re grew with similar efficiencies in MA104 cells, indicating that the atypical gene 7 RNA did not alter viral growth kinetics as measured by progeny formation (Fig. 1b). Analysis of plaques formed by SA11-4Fg7re indicated they were less well defined than those of SA11-4F during earlier times of the plaque assay (data not shown).

**Gene 7 sequence duplication in SA11-4Fg7re**

Sequence analysis showed that the SA11-4F gene 7 RNA was 1105 nt in length, differing by only a single nucleotide from the gene 7 sequence reported for SA11-C4 by Mossel & Ramig (2002) (Fig. 2a). Like earlier reports (Both et al., 1984; Mossel & Ramig, 2002), our sequence results indicate that SA11-4F gene 7 RNA ends with the sequence UUGGCC instead of the typical consensus sequence UUGGACC. The SA11-4F gene 7 RNA contains two potential AUG initiation codons for the NSP3 ORF. However, only the second (residues 35–37) is common to all gene 7 RNAs and, according to Kozak’s rules (Kozak, 1981), is better configured to serve as the initiation codon (Both et al., 1984; Gault et al., 2001; Ward et al., 1984). From the second AUG, the NSP3 ORF would produce a protein of 312 aa. Sequence analysis of SA11-4Fg7re indicated that its gene 7 RNA was 1768 nt in length and contained a rearrangement resulting from a 663 nt head-to-tail sequence duplication of residues 100–763 of the SA11-4F gene 7 RNA. Like the SA11-4F gene 7 RNA, the rearranged gene 7 RNA of SA11-4Fg7re contains two in-frame AUG codons. Based on the prediction that the second AUG represents the authentic site of initiation, the resulting NSP3 product (NSP3m) of SA11-4Fg7re would have a length of 533 aa. The rearrangement junction in the NSP3m protein occurs between aa 243 and 244, which marks the beginning of the duplicated portion of NSP3. The duplicated portion corresponds to aa 23–312 of the wild-type protein (Fig. 2b).

**Predicted protein domains of SA11-4F NSP3 and SA11-g7re NSP3m**

X-ray crystallography has shown that NSP3 self assembles into a homodimer that contains a single N-terminal RNA-binding pocket and two C-terminal eIF4G-binding pockets (Deo et al., 2002; Groft & Burley, 2002). These regions are structurally and functionally discrete from one another and are molecularly bridged by an extended coiled-coil (Deo et al., 2002; Groft & Burley, 2002; Piron et al., 1999) (Fig. 2b). The coiled-coil region includes an interactive site for
RoXaN I, a cellular protein of unknown function (Harb et al., 2008; Vitour et al., 2004). By extrapolation from information on the structure and function of wild-type NSP3, NSP3m can be predicted to form an intact N-terminal RNA-binding pocket and the two intact C-terminal eIF4G-binding pockets (Fig. 2b). The sequence rearrangement introduces two complete copies of the bridging coiled-coil domain in NSP3m, one that abuts the N-terminal RNA-binding pocket and the other abuts the C-terminal eIF4G-binding pockets. The COILS (MacStripe) software program (Lupas et al., 1991) indicates that there is a high probability that both coiled-coils will form in NSP3m (data not shown). The duplicated nature of the coiled-coil domain implies that NSP3m also has two copies of the RoXaN-binding domain. Situated centrally within the NSP3m protein, between the two coiled-coil domains, is a truncated version of the RNA-binding domain, which is unlikely to retain function as it lacks residues that support and line the RNA-binding pocket (data not shown).

**SA11-4Fg7re expresses mutant NSP3**

To verify that gene 7 of SA11-4Fg7re expressed the predicted larger-sized NSP3 product, MA104 cells were infected with SA11-4F and SA11-4Fg7re and maintained in the presence of 35S-labelled amino acids. Proteins in the lysates were treated at 95 °C with SDS and β-mercaptoethanol, resolved by SDS-PAGE and detected by autoradiography (Fig. 3a). The NSP3 protein produced by the wild-type SA11-4F virus had an apparent molecular mass of 36 kDa. This band was absent in SA11-4Fg7re-infected cells. Instead, a radiolabelled band of the predicted size of NSP3m (62 kDa) was present in the SA11-4Fg7re-infected cells. The 36 and 62 kDa bands were confirmed to be NSP3 products by Western blot analysis using NSP3 antiserum (Fig. 3b). Other than the NSP3 proteins, no differences were noted in the types or quantities of viral proteins made in SA11-4F- and SA11-4Fg7re-infected cells.

**Mutant NSP3 undergoes dimerization**

To determine whether NSP3m retained the capacity to dimerize, 35S-labelled lysates from SA11-4F- and SA11-4Fg7re-infected cells were treated with SDS and β-mercaptoethanol at 23 °C. Afterwards, the samples were analysed by gel electrophoresis and autoradiography and by Western blot assay using NSP3 antisera. The results showed that the SA11-4F sample treated at 23 °C produced a 72 kDa protein band on gels, a size approximating that of an NSP3 dimer (Fig. 4a). Western blot analysis confirmed that the 72 kDa band consisted of NSP3 (Fig. 4b). This band was not produced upon electrophoresis of the 95 °C heat-treated sample, where all NSP3 was detected in the 36 kDa monomeric form. Also the 72 kDa band was not detected in gels of the SA11-4Fg7re sample, treated at 23 or 95 °C. Instead, the SA11-4Fg7re sample treated at 23 °C produced a 124 kDa band, a size approximately twice that of the NSP3m monomer (62 kDa) (Fig. 4a, b). The putative NSP3m-dimer was not present in the 95 °C-treated sample, but instead existed only in the monomeric 62 kDa form. These results suggest that NSP3m, like the wild-type protein, retained the capacity to undergo dimerization in the infected cell. Consistent with earlier reports (Clapp & Patton, 1991; Gorziglia et al., 1985; Sabara et al., 1987), VP6 trimers were present in SA11-4F and SA11-4Fg7re samples that were incubated with SDS and β-mercaptoethanol at 23 °C, but absent in these samples if they were incubated at 95 °C instead (Fig. 4a, c).

**NSP3m binds RoXaN and eIF4G**

Previous co-immunoprecipitation assays have shown that wild-type NSP3 can interact with transiently expressed RoXaN and eIF4G. The co-immunoprecipitation assays with wild-type NSP3 and the mutant NSP3m with RoXaN and eIF4G are shown in Fig. 4a, b. The results show that NSP3m can bind RoXaN and eIF4G, similar to wild-type NSP3.
RoXaN in RV-infected cells (Vitour et al., 2004). To test whether NSP3m retained RoXaN-binding activity, we transfected HEK293T cells with pCI vectors encoding Flag-tagged full-length RoXaN (F-RoXaN) and NSP3 or NSP3m. Twenty-four hours later, immunoprecipitates were prepared from the transfected cells by incubation with Flag antibody, which were then analysed by Western blot assay using NSP3 antiserum (Fig. 5a). The results showed that wild-type NSP3 (lane 9) and NSP3m (lane 10) were present in the Flag precipitates. Wild-type NSP3 and NSP3m were not present in precipitates prepared in the absence of Flag antibody (lanes 6–7). These data indicate that NSP3m, like wild-type NSP3, retained a functional RoXaN-binding domain. The origin of the lower band (*) in lanes 9 and 10 (Fig. 5a) is not clear, but since equivalent faint bands are also present in lanes 7 (NSP3m only) and 8 (RoXan), it probably does not represent RoXaN or NSP3 material.

Interestingly, our results turned out differently than those presented in Fig. 5(a) when we reversed our approach for detecting RoXaN–NSP3 complexes (Fig. 5b). In this case, immunoprecipitates were prepared from the transfected cells by incubation with NSP3 antisera, which were then analysed by Western blot assay using Flag antibody. The results showed that although the precipitates included wild-type NSP3 or NSP3m, they lacked Flag-tagged RoXaN for reasons that are unclear (lanes 9–10). One possibility is that the large size of RoXaN and its interaction with other cellular proteins, creates RoXaN–NSP3 complexes in which the epitopes for the NSP3 antibody are sequestered.

Co-immunoprecipitations assays have shown that NSP3 interacts with endogenous eIF4G in RV-infected cells (Piron et al., 1998). To determine whether NSP3m could interact with eIF4G, NSP3 antiserum was used to prepare immunoprecipitates from HEK293T cells containing transiently expressed NSP3 or NSP3m. Analysis of the precipitates by Western blot assay revealed that both contained eIF4G, indicating that like wild-type NSP3, NSP3m retained eIF4G-binding activity (Fig. 5c, lanes 6–7). Importantly, although eIF4G was detected in the NSP3m precipitate (lane 7), its level was lower than that detected in the NSP3 precipitate (lane 6). The same pattern of bands was also observed in NSP3 precipitates from cells expressing NSP3m (lane 8).

### Figure 2

Schematic illustration of the gene 7 RNAs and NSP3 products of SA11-4F and SA11-4Fg7re. (a) Location of the UTRs (solid bar) and ORF (box) in the gene 7 RNAs of SA11-4F and SA11-4Fg7re. The gene 7 RNA of SA11-4Fg7re contains a head-to-tail in-frame sequence duplication that begins at residue 763. Nucleotide positions are indicated. Positions given in parentheses for the gene 7 RNA of SA11-4Fg7re refer to the corresponding positions of the gene 7 RNA of SA11-4F. (b) Location of domains in wild-type NSP3 and the mutant NSP3m. NSP3 contains an N-terminal RNA-binding domain (RNA-BD), central coiled-coil (Coil) and RoXaN-binding (RoXaN) domains, and a C-terminal eIF4G-binding domain (4G-BD). NSP3m expressed by SA11-4Fg7re retains an intact N-terminal RNA-BD and an intact C-terminal eIF4G-binding domain. Amino acid positions are numbered. Those numbers shown below the NSP3m schematic refer to the corresponding positions of wild-type NSP3.

### Figure 3

Production of wild-type NSP3 and NSP3m by SA11-4F and SA11-4Fg7re, respectively. (a) 35 S-labelled proteins in mock- or virus-infected cell lysates at 9 h p.i. were detected by gel electrophoresis and autoradiography. (b) NSP3 and NSP3m in infected cell lysates were identified by immunoblot assay using αNSP3 antiserum. Wild-type NSP3 and NSP3m are indicated with asterisks and some viral proteins are labelled in (a).
was observed upon analysis of immunoprecipitates recovered from cell lysates containing transiently expressed NSP3 and RoXaN, or NSP3m and RoXaN. Again, the level of eIF4G detected in the NSP3m precipitate (lane 10) was lower than that detected in the NSP3 precipitate (lane 9). These data suggest that the structure of NSP3m is such that eIF4G has restricted access to the eIF4G-binding site or that the eIF4G-binding site is misfolded, weakening its interaction with its ligand. Alternatively, the mutant NSP3m structure may cause the protein to mislocalize in the cytoplasm to regions where pools of eIF4G levels are low. As shown by analysis of cell lysates containing only transiently expressed F-RoXaN (lane 8), eIF4G was not recovered in precipitates that were processed without NSP3 antisera, pointing to the specificity of the eIF4G–NSP3 and eIF4G–NSP3m interactions.

**Effect of NSP3m on protein expression**

In our transient expression experiments, NSP3, NSP3m and F-RoXaN were encoded by polyadenylated transcripts produced from transfected pCI vectors. We observed in these experiments that NSP3 was produced at lower levels than NSP3m (Fig. 5a, lanes 1–2) and that F-RoXaN was produced at lower levels when co-expressed with NSP3 than with NSP3m (Fig. 5b, lanes 1–2). One interpretation of these results is that NSP3, by disrupting eIF4G–PABP complexes, may be interfering with efficient translation of its own polyadenylated RNA as well as those of others (e.g. F-RoXaN) in transfected cells. This interpretation could be extended to suggest that NSP3m, relative to NSP3, is defective in the ability to interfere with the translation of polyadenylated RNA. To test this interpretation experimentally, HEK293T cells were co-transfected with pCI vectors that directed expression of NSP3, NSP3m, or no NSP3 and with the pGL3 vector, which produces polyadenylated luciferase mRNA. Analysis of luciferase levels at 24 h post-transfection showed that luciferase expression was four- to fivefold lower in transfected cells expressing NSP3 than those expressing either NSP3m or no NSP3 (Fig. 6). These results support the idea that NSP3 interferes with the translation of polyadenylated mRNAs and that NSP3m is defective in this activity.

**SA11-4Fg7re does not induce nuclear accumulation of PABP**

To contrast the subcellular localization of wild-type NSP3 and NSP3m, MA104 cells were infected with SA11-4F or SA11-4Fg7re, co-immunostained with NSP2- and NSP3-specific antisera and visualized by immunofluorescence (Fig. 7a). The results showed that wild-type NSP3 and NSP3m were similarly distributed throughout the cytoplasm of infected cells, with no evidence of nuclear accumulation. The pattern of NSP2 distribution indicated that the size and location of viroplasms were also similar in SA11-4F- and SA11-4Fg7re-infected cells. Neither wild-type NSP3 nor NSP3m accumulated in viroplasms.

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**Fig. 4. Dimerization of NSP3 and NSP3m.** (a) 
\(^{35}\)S-labelled proteins from mock-infected or SA11-4F- or SA11-4Fg7re-infected cells were incubated at 23 or 95 °C in sample buffer containing SDS and β-mercaptoethanol and detected by gel electrophoresis and autoradiography. Positions of monomeric and dimeric NSP3 (monNSP3, ‘*’ and diNSP3, ‘**’), monomeric and dimeric NSP3m (monNSP3m, ‘*’ and diNSP3m, ‘**’), and monomeric and trimeric VP6 (monVP6, ‘●’ and triVP6, ‘● ● ●’) are indicated. Positions of NSP3 and NSPm monomers and dimers (b) and VP6 monomers and trimers (c) in (a) were verified by immunoblot assay with αNSP3 and αVP6 antisera.
To determine whether NSP3m induced the nuclear localization of PABP, SA11-4F- and SA11-4Fg7re-infected MA104 cells were immunostained with NSP3- and PABP-specific antisera and visualized by immunofluorescence (Fig. 7b). In mock-infected cells, PABP was predominantly contained in the cytoplasm, where it was distributed diffusely. In cells infected with SA11-4F, a significant relocalization of PABP occurred, with large amounts of PABP concentrating in multiple extra-nucleosomal inclusions of the nucleus. In contrast, PABP remained cytoplasmic in SA11-4Fg7re-infected cells. This result suggests that NSP3m was not efficient in evicting PABP from the eIF4G cap-binding complexes in the cytosol, thus preventing relocation of PABP to the nucleus.

**DISCUSSION**

This is the first description of an RV that fails to induce the relocalization of PABP to the nucleus. The fact that SA11-4F and SA11-4Fg7re grow to similar titres in cell culture indicates that PABP relocation is not an essential feature of the RV life cycle. The failure of SA11-4Fg7re infection to induce PABP relocalization is associated with a sequence rearrangement in its gene 7 ORF that causes the virus to encode a partially duplicated NSP3 product (NSP3m). A primary role of PABP is to assist the shuttling of host mRNAs from the nucleus to the cytoplasm. The fact that PABP accumulates in the nucleus of SA11-4F-infected cells indicates that NSP3 may be interfering with host mRNA shuttling and, thereby,
preventing their movement to the cytoplasm where they could be translated. In contrast, the failure of NSP3m to trigger nuclear accumulation of PABP in SA11-4Fg7re-infected cells suggests that PABP retains the capacity to shuttle host mRNAs to the cytoplasm, thus favouring their translation. The contrasting effects of NSP3 and NSP3m on PABP nuclear accumulation and possibly mRNA shuttling may account for the low level of luciferase expression detected in cells co-transfected with vectors for NSP3 and luciferase mRNA versus the high level of luciferase expression detected in cells co-transfected with vectors for NSP3m and luciferase mRNAs (Fig. 6).

Sequencing indicates that the NSP3m protein has a complete N-terminal RNA-binding domain and a complete C-terminal eIF4G-binding domain. NSP3 and NSP3m are made at similar levels in infected cells and dimerization assays indicate that NSP3m has functional coiled-coil domains that allow the protein to form dimers. These properties imply that NSP3m dimers may retain affinity for RNA, eIF4G and RoXaN. Indeed, co-immunoprecipitation assays confirm that NSP3m can bind RoXaN. Given that the RoXaN-binding domain is positioned within the coiled-coil domain of NSP3, NSP3m with its duplicated coiled-coil domain may bind two copies of RoXaN. Our analysis indicates that, compared with NSP3, NSP3m has decreased capacity to interact with eIF4G. This difference may explain why NSP3m fails to induce PABP nuclear localization in SA11-4Fg7re-infected cells; the mutant protein lacks the ability to disrupt the cellular eIF4G–PABP complexes that gives rise to the PABP that accumulates in the nucleus. It is

Fig. 7. Localization of NSP3 products and PABP in SA11-4F- and SA11-4Fg7re-infected cells. The intracellular distribution of NSP2 (red) and NSP3 (green) in SA11-4F- or SA11-4Fg7re-infected cells (a) and NSP3 (green) and PABP (red) in mock, SA11-4F- or SA11-4Fg7re-infected MA104 cells (b) at 12 h p.i. was determined by confocal immunofluorescence assay using protein-specific antisera. Nuclei were stained with DAPI.
not obvious why the NSP3m dimer, in contrast to NSP3, interacts inefficiently with eIF4G. The most obvious difference between NSP3 and NSP3m is the distance between their N-terminal RNA-binding and C-terminal eIF4G-binding domains. Specifically, the length of the ‘spacer’ between the two terminal domains of the mutant protein is more than 200 aa greater than that of the wild-type protein (Fig. 2b). Perhaps, the inability of NSP3m to induce PABP relocalization may result from the longer ‘spacer’ of the mutant protein preventing its terminal RNA- and eIF4G-binding domains from appropriately working in concert with each another to disrupt eIF4G–PABP complexes. It is also possible that NSP3m, by binding two copies of RoXaN, forms protein complexes that cannot interact appropriately with eIF4G.

RVs have been isolated with intragenic sequence rearrangements in one or more genome segments, including those encoding VP6, NSP1, NSP2, NSP3, NSP4 and NSP5 (Ballard et al., 1992; Gault et al., 2001; Hundley et al., 1985; Kojima et al., 1996, 2000; Scott et al., 1989; Shen et al., 1994; Tian et al., 1993). In most cases, these rearrangements result from a head-to-tail sequence duplication that initiates near the junction of the ORF and 3′-UTR. Thus, such rearrangements typically do not affect the coding capacity of the genome segment. However, there are notable exceptions. For instance, several RVs have been isolated with sequence rearrangements that initiate in the NSP1 ORF of the gene 5 RNA (Hua & Patton, 1994; Patton et al., 2001; Taniguchi et al., 1996; Tian et al., 1993). As a result, these viruses encode defective forms of NSP1 that lack the capacity to subvert interferon signalling (Arnold & Patton, 2011; Barro & Patton, 2005; Graff et al., 2007). The fact that viruses with such gene 5 rearrangements still grow to high titre in cell culture has been used as evidence that NSP1 has a non-essential role in virus replication.

Two categories of RVs have been isolated with gene 7 sequence rearrangements (Fig. 8). The largest category includes viruses with gene 7 rearrangements that begin after the NSP3 ORF (Fig. 8a) (Alam et al., 2008; Cao et al., 2008; Gault et al., 2001; Kojima et al., 2000; Yuan et al., 2006). Hence, these viruses encode wild-type NSP3. The majority of such variant RVs have been recovered from virus pools serially passaged at a high m.o.i. in cell culture. The second category includes RVs with gene 7 rearrangements that initiate within the NSP3 ORF, and thus cause

![Fig. 8. Schematic illustrating the two classes of rearranged gene 7 RNAs expressing NSP3.](image-url)
the virus to encode mutant forms of NSP3 (Fig. 8b). The SA11-4Fg7re variant described in this report is only the second member identified in this category. The other member (M3-7RA) was recovered, along with its wild-type homologue (strain M), from an infant with combined immunodeficiency syndrome (Gault et al., 2001). Although this suggests that M3-7RA is viable in humans, there remains a possibility that the variant may not grow in immunocompetent children, where virus replication may be more dependent on a fully functional NSP3 to promote efficient viral protein expression. Like SA11-4Fg7re, M3-7RA has been shown experimentally to express a long mutant form of NSP3 in cell culture. It has not been determined whether or not PABP relocalization occurs in M3-7RA-infected cells.

The mechanism of intragenic sequence rearrangement remains uncertain, although multiple hypotheses have been put forth (Gault et al., 2001; Kojima et al., 1996; Matthijssens et al., 2006). However, the recent identification of virus particles containing chimeric NSP2–NSP5 genomic dsRNAs suggests that sequence rearrangements result from the viral RNA polymerase switching from its initial RNA template to a second co-occupying RNA template contained within the catalytic core (Cao et al., 2008). SA11-4Fg7re represents yet another example of an RV with a rearranged genome segment generated by serial passage at high m.o.i. It is interesting that most such mutant RVs involve segments (i.e. genes 5 and 7) encoding proteins apparently not required for growth in cell culture (Kojima et al., 2000; Patton et al., 2001; Tian et al., 1993). Genome segments with rearrangements have a selective packaging advantage over cognate wild-type RNAs during serial passage of RVs in cell culture (Troupin et al., 2010). Perhaps this is due to genome rearrangements causing a duplication of packaging signals in the RNA molecule. However, since such rearrangements in some cases do not involve UTR sequences, the packaging signals that are in play would have to be contained in the ORF.

METHODS

Cells, viruses and antibodies. Embryonic monkey kidney cells (MA104) were maintained in M199 (Invitrogen) supplemented with 5% FBS. Human HEK293T cells were maintained in Dulbecco’s modified Eagle medium (Invitrogen) supplemented with 10% FBS and 1% non-essential amino acids. SA11-4F (G3P[3]) and SA11-4Fg7re were propagated and titrated in MA104 cells (Arnold et al., 2009). The SA11-4Fg7re variant was isolated by triple-plaque 4Fg7re were propagated and titrated in MA104 cells (Arnold et al., 2009). Polyclonal NSP3 antiserum was produced by immunizing guinea pigs with VP6 recovered from DxRRV double-layered particles treated with 1 M CaCl2 (Patton & Chen, 1999). mAb anti-Flag was purchased from Sigma-Aldrich (cat. no. F1804) and polyclonal anti-eIF4G antibody was purchased from Cell Signaling Technology (cat. no. 2498). mAb anti-PABP (10E10) was purchased from Santa Cruz Biotechnology, Inc. (cat. no. sc-32318). Goat anti-guinea pig IgG conjugated to Alexa 488 and goat anti-mouse IgG conjugated to Alexa 594 were purchased from Molecular Probes. Peroxidase-labelled goat anti-mouse IgG, anti-rabbit IgG and anti-guinea pig IgG were purchased from KPL, Inc.

Radiolabelling viral proteins. MA104 cells were mock-infected or infected with trypsin-activated RV at an m.o.i. of 10. After 1 h, the inoculum was replaced with 80% methionine-cysteine-free MEM containing 25 µCi [35S]-amino acids ml-1. At 9 h post-infection (p.i.), the cells were recovered and resuspended in hypotonic buffer (3 mM NaCl, 3 mM HEPES-NaOH, pH 8.0, 0.5 mM MgCl2) containing 1 × Complete protease inhibitor according to the manufacturer’s protocol (Roche). The cells were lysed by Dounce homogenization under detergent-free conditions; membrane debris and nuclei were removed by centrifugation for 10 min at 10,000 g. Lysates were incubated with sample buffer containing SDS and β-mercaptoethanol at room temperature or 95°C and resolved by electrophoresis on Novex 10% or 4–20% Tris-glycine polyacrylamide gels (Invitrogen). Radiolabelled viral proteins were detected by autoradiography.

Vectors. The NSP3 ORF of SA11-4F gene 7 RNA and the NSP3m ORF of SA11-4Fg7re gene 7 RNA were amplified by RT-PCR with Superscript II and Platinum Taq polymerases (Invitrogen) and the sense primer 5′-CGGTCGACCTGTCGAAGATGCTGACG-3′ (NhI site underlined, ORF start codon bold) and the antisense primer 5′-CTAGCGGAGGTGCTGACG-3′ (EcoRI site underlined, ORF stop codon bold). The cDNAs were digested with Nhel and EcoRI and ligated into pC1-neo vector (Promega) digested with the same enzymes. Gene sequences of the resulting vectors (pCI-NSP3 and pCI-NSP3m) were verified by sequencing.

The ORF of human ZC3H7B (RoXaN I; GenBank accession no. BC167846) in the Gateway Entry vector pENTR223.1 (Invitrogen) was purchased from Open Biosystems. The RoXaN-coding gene in pENTR223.1 was inserted into the destination vector pCDNA-DEST40 by recombination using LR Clonase II (Invitrogen). A Flag tag was inserted into the resulting vector by outward PCR using the sense primer 5′-CATGGACAGACTATAGGACGATGATGACGATGGTAGC-3′ (Flag-tag underlined, RoXaN ORF start codon bold) and the antisense primer 5′-GGGTTATGAGAGGCAGAAACGGATTAGC-3′ (Flag-tag underlined, RoXaN ORF stop codon bold) and the antisense primer 5′-GATCTTTTCAAGCTCAGAG-3′ (Flag-tag underlined, RoXaN ORF stop codon bold) and the antisense primer 5′-GATCTTTTCAAGCTCAGAG-3′ (Flag-tag underlined, RoXaN ORF stop codon bold) and the antisense primer. The PCR product was self-ligated to generate pCMV-Flag-RoXaN. The pGL3-Control Vector (Promega) includes SV40 promoter and enhancer elements that direct the transcription of polyadenylated luciferase mRNA.

Sequence analysis of gene 7 dsRNA. RV dsRNAs were purified from virus by phenol/chloroform extraction and detected by electrophoresis on 10% polyacrylamide gels and staining with ethidium bromide. cDNAs of gene 7 dsRNAs were amplified using a OneStep RT-PCR kit (Invitrogen) and the gene-specific terminal primers 5′-GGGTTATGAGAGGCAGAAACGGATTAGC-3′ and 5′-CACATACAGCGCCCTTATAGC-3′. Full-length RT-PCR products were gel purified with the QIAquick Gel Extraction kit (Qiagen) and ligated into the plasmid pcR2.1 using the TOPO TA cloning kit (Invitrogen). Clones were sequenced by using an ABI Prism BigDye v3.1 terminator cycle sequencing kit and analysed with an ABI Prism 310 Genetic Analyzer (Applied Biosystems).
gene-specific terminal primers to prepare the gene 7 cDNAs precluded \textit{de novo} identification of the first 23 and last 24 residues of the viral RNAs. The sequence of these residues for the SA11-4F gene 7 RNA were determined by 3' RACE (Invitrogen). The gene 7 sequence of SA11-4F is provided under GenBank accession no. GU550506 and that of SA11-4Fg7re is available under GenBank accession no. EU934821.

**Luciferase assay.** HEK293T cells were plated in 12-well tissue culture plates at a density of 1 × 10^5 cells per well. The following day, cells were transfected with 0.5 µg pGL3-Control vector and 1.0 µg pcl-NSP3, or pcl-NSP3m by using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). At 24 h post-transfection, cells were washed with PBS and then lysed in 100 µl of the Cell Culture Lysis Reagent (Promega). Cell debris was removed by brief centrifugation and 75 µl of the cell lysate was placed in a 96-well plate. After addition of 75 µl Dual-Glo Luciferase Reagent (Promega) to each well, the plate was incubated for 10 min. Luminescence was measured using a SpectraMax M5 microplate reader (Molecular Devices).

**Western blot analysis.** Proteins were treated with SDS and β-mercaptoethanol at 95 °C, resolved by electrophoresis on Novex Tris-glycine gels (Invitrogen) and transferred onto nitrocellulose membranes. Afterwards, the membranes were blocked with PBS containing 5 % Carnation powdered milk and 0.1 % Tween-20 and then incubated with guinea pig VP6 or NSP3 antisera in PBS for 1 h at 30 °C and then washed in PBS containing 1 % milk and 0.1 % Tween-20. Blots were washed with PBS and incubated with HRP-conjugated secondary antibodies in PBS containing 1 % milk and 0.1 % Tween-20. Blots were developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce) and exposed to BioMax MR film (Kodak).

**Immunoprecipitation.** Transfected cells were lysed by incubation for 30 min on ice in non-denaturing lysis buffer (20 mM Tris/HCl, pH 8.0, 137 mM NaCl, 10 % glycerol, 1 % Nonidet P-40) containing Complete protease inhibitor (Roche). After centrifugation at 13 000 × g to pellet cellular debris, lysates were transferred to new tubes. Immunoprecipitating antibodies were incubated with magnetic protein A Dynal Beads (Invitrogen) in 0.1 M sodium phosphate buffer (pH 8.0). Bound antibody was cross-linked to beads with dimethyl pimelimidate (Pierce) in 0.2 M triethanolamine (pH 8.2). Beads cross-linked with antibody were added to cell lysates, which were incubated with rotation for 1 h at room temperature. Immunoprecipitated proteins were washed in PBS and proteins were eluted with Novex Tris-Glycine SDS Sample buffer, 2 × (Invitrogen) or 0.1 M glycine (pH 3.0).

**Immunofluorescence analysis.** MA104 cells were grown on glass coverslips and infected with RV at an m.o.i. of 10. At 12 h p.i., the cells were fixed with 4 % formaldehyde in PBS for 30 min at room temperature and then washed with PBS. The cells were incubated in PBS containing 5 % γ-globulin-free BSA for 15 min at room temperature and then washed in PBS containing 1 % Triton X-100. The cells were then incubated with guinea pig NSP3 antisera, or mouse anti-NSP2 or -PABP mAbs in PBS containing 3 % BSA for 1 h at 30 °C. The cells were washed in PBS containing 1 % Triton X-100, followed by co-incubation with goat anti-guinea pig IgG and anti-mouse IgG conjugated to Alexa 488 and Alexa 594 (Molecular Probes), respectively, in PBS containing 3 % BSA for 1 h at 30 °C. Cells were washed in PBS and coverslips were mounted with ProLong Antifade Reagent containing 4,6-diamino-2-phenylindole (DAPI) (Invitrogen). Fluorescence was detected with a Leica inverted confocal microscope; images were processed using Photoshop CS2.

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**REFERENCES**


