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Protocols

Development of an optimized RNA-based murine norovirus reverse genetics system

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Murine norovirus (MNV), identified in 2003, is the only norovirus which replicates efficiently in tissue culture and as a result has been used extensively as a model for human noroviruses, a major cause of acute gastroenteritis. The current report describes the generation of a new approach to reverse genetics recovery of genetically defined MNV that relies on the transfection of *in vitro* transcribed capped RNA directly into cells. The use of the recently developed ScriptCap post-transcriptional enzymatic capping system, followed by optimized Neon mediated electroporation of the highly permissive RAW 264.7 cells, resulted in the rapid and robust recovery of infectious MNV. Transfection of cells capable of supporting virus replication but not permissive to virus infection, namely human or hamster kidney cells, also resulted in robust recovery of infectious virus without subsequent amplification by multiple rounds of re-infection. This latter system may provide a reproducible method to measure the specific infectivity of mutant norovirus RNA allowing the accurate quantitation of the effect of mutations on norovirus replication.

1. Introduction

Since the first demonstration that *in vitro* transcribed RNA from a cDNA clone of a positive strand RNA virus (Polio) was infectious in tissue culture (Racaniello and Baltimore, 1981), reverse genetics has proven to be an invaluable approach to studies on many aspects of virus replication and pathogenesis. In many cases, it has allowed an unprecedented ability to examine the effect of mutations not only on virus replication in tissue culture, but also on pathogenesis in the natural host. For some organisms this has led to the development of rationally attenuated viruses (Cavanagh et al., 2007) or improved vaccine candidates (Macadam et al., 2006).

Murine norovirus (MNV), a member of the *Caliciviridae* family of small positive stranded RNA viruses, was first reported in 2003 (Karst et al., 2003) and still represents the only norovirus which replicates efficiently in tissue culture (Wobus et al., 2004). The discovery of MNV has allowed an unprecedented analysis of the role of viral sequences in norovirus translation, replication and pathogenesis in the natural host. As a result, MNV is used widely as a model system for the human noroviruses, a major cause of viral gastroenteritis in man (Wobus et al., 2006). The murine norovirus genome contains four reading frames (Fig. 1A); ORF1 encodes a large polyprotein which is cleaved co- and post-translationally to produce the viral non-structural proteins required for viral genome replication (NS1-7) (Sosnovtsev et al., 2006); ORF2 encodes the major capsid protein VP1; ORF3 encodes a minor structural protein whereas ORF4 encodes a protein of unknown function (Sosnovtsev et al., 2006).

A reverse genetics approach for MNV described previously relies on the transfection of a full-length cDNA construct of MNV into cells infected previously with fowlpox virus expressing T7 RNA polymerase (FPV-T7) (Chaudhry et al., 2007). This system was used subsequently to address many aspects of the norovirus life cycle including the identification of RNA structures important for norovirus replication (Simmonds et al., 2008), as well as mapping virulence determinants in the viral capsid protein (Bailey et al., 2008) and the 3′ untranslated region of the viral genome (Bailey et al., 2010). An RNA polymerase I promoter based reverse genetics system for the recovery of MNV has also been described (Ward et al., 2007), although published yields appear to be greater than 10-fold lower than those obtained using FPV-T7; 10^3 plaque forming units/ml versus $>5 \times 10^4$ TCID50 per 35 mm dish for the Pol-I and FPV-T7 based systems respectively.

During the course of previous studies to generate a reverse genetics system (Chaudhry et al., 2007), the use of capped *in vitro* synthesised MNV RNA was examined as a possible reverse genetics approach as this has proven effective for other members of the *Caliciviridae*; both feline calicivirus (Sosnovtsev and Green, 1995) and porcine enteric calicivirus (Chang et al., 2005).

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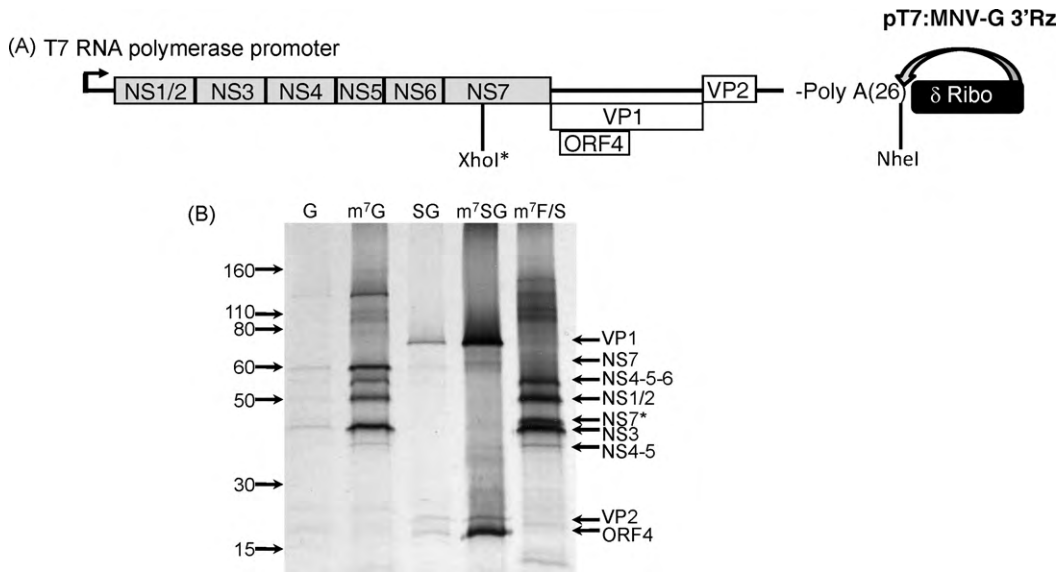

Fig. 1. Murine norovirus full-length cDNA clone and translation of *in vitro* synthesised RNA. (A) Schematic of the infectious cDNA clone used in this study highlighting the positions of the four open reading frames, the T7 RNA polymerase promoter and the position of the 3′ ribozyme sequence. The asterisk highlights the position of the frame shift used to generate the mutated cDNA clone pT7:MNV-G 3′Rz F/S. (B) *In vitro* translation of *in vitro* transcribed murine norovirus genomic (G) or subgenomic RNA (SG). RNA was *in vitro* transcribed as described in Section 2.3 and in some cases enzymatically capped, highlighted by the prefix m^7, prior to translation in rabbit reticulocyte lysates. Samples were resolved subsequently by 15% SDS-PAGE prior to exposure to a phosphoimager screen. Note that the protein assignments are based on the nomenclature proposed previously (Sosnovtsev et al., 2006). NS7* represents the truncated NS7 product generated as a result of the frame shift introduced in pT7:MNV-G 3′Rz F/S.

However infectious virus was not recovered by transfection of capped *in vitro* transcribed MNV RNA into a number of efficiently transfected cell lines, including human 293T and hamster BHK cells, which although not permissive to infection by MNV viral particles, resulted in robust virus release when transfected with purified VPg-linked viral RNA (Chaudhry et al., 2007). In addition, transfection of *in vitro* transcribed capped viral RNA into the highly permissive, but more refractile to transfection RAW 264.7 murine macrophage cell line also failed to result in virus recovery. However, in this case, difficulties in the efficient delivery of RNA may have had a major impact on the ability to recover virus.

The current report describes the generation of an efficient and robust method for recovery of genetically defined murine norovirus from *in vitro* transcribed, post-transcriptionally capped RNA by transfection of several different cell types. This method displays >10-fold increase in virus yield when compared to the methods established previously and provides an additional approach with which to accurately quantify the effect of mutations within the norovirus genome on virus replication.

2. Materials and methods

2.1. Cells

Human embryonic kidney (293T) and murine macrophage cells (RAW 264.7) were obtained from ATCC and maintained in DMEM containing 10% foetal calf serum (FCS) at 37 °C with 10% CO_2. BHK cells engineered to express T7 RNA polymerase (BSR-T7), as described previously (Buchholz et al., 1999), were obtained from Karl-Klaus Conzelmann (Ludwig Maximilians University, Munich, Germany) and maintained as described for 293 cells with the inclusion of 0.5 mg/ml G418. Note that BSR-T7 cells were used for this study simply due to their improved growth characteristics compared to BHK parental cells and the expression of T7 RNA polymerase in these cells has no effect on virus recovery as similar results are obtained using BHK (data not shown).

2.2. Plasmids and primers

pT7:MNV 3′Rz containing the wild type MNV-1 sequence under the control of a truncated T7 RNA polymerase promoter was described previously (Chaudhry et al., 2007). A modified version of this in which a frame shift was introduced into the NS7 region of the ORF1 open reading frame (pT7:MNV 3′Rz F/S) has also been described previously (Chaudhry et al., 2007). To generate a cDNA template for the synthesis of MNV subgenomic RNA, the PCR primers IGIC18 (TAATACGACTCACTATAGGGGTGAATGAGGATGAGTGATGGC) and 7400R (TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTAAAATGCATCTAACTACCACAAAG) were used to amplify the viral subgenomic region, introducing a 5′ T7 RNA polymerase promoter and 30 nucleotides long 3′ poly-A tail.

2.3. In vitro transcription, RNA capping and RNA purification

Typically, transcription reactions contained 200 mM Hepes pH 7.5, 32 mM magnesium acetate, 40 mM DTT, 2 mM spermidine, 7.5 mM of each NTP, 25 ng/μl of linearised DNA template and 50 μg/ml of T7 RNA polymerase. Reactions were incubated at 37 °C for 2–7 h, treated with DNase at a final concentration of 0.1 unit/μl (New England Biolabs) then precipitated using lithium chloride, final concentration 2.5 M. RNA was resuspended in RNA storage solution (Ambion) and stored at −20 °C until required. The cDNA clones pT7:MNV 3′RZ or pT7:MNV F/S 3′RZ were linearised with NheI prior to *in vitro* transcription. RNA transcripts produced in this manner resulted in the inclusion of GCUAG at the 3′ end of the viral transcript due to the *NheI* overhang. RNA was capped using the ScriptCap system from Epicentre according to the manufacturer's instructions. Briefly, up to 75 μg of RNA was denatured by heating to 65 °C for 10 min prior to rapid chilling on ice. The capping reaction was then set up by the addition of 10 μl of 10× capping buffer, 10 μl of 10 mM GTP, 0.5 μl of 20 mM S-adenosyl methionine, 2.5 μl of Scriptguard and 4 μl of ScriptCap enzyme mix in a total reaction volume of 100 μl. The reaction was incubated at 37 °C for 1 h then the RNA precipitated using lithium chloride. RNA was washed