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A novel reverse genetics system for human norovirus

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Abstract

Human noroviruses cause significant morbidity, mortality, and economic losses worldwide. The inability to grow human noroviruses in cell culture has hampered our collective understanding of virus–host interactions and development of therapeutics. A newly described single-plasmid reverse genetics system for noroviruses has the potential to facilitate basic and applied research.

Keywords

norovirus reverse genetics system; generation of reporter-tagged viruses

Noroviruses are positive-sense, single-stranded RNA viruses in the *Caliciviridae* family [1]. The genus is divided into six genogroups (G), three of which infect humans (GI, II, and IV), one infects mice (GV), and the rest infect other mammals, e.g., canines (GIV and GVI) and bovines (GIII) [1]. Human noroviruses cause the majority of viral gastroenteritis worldwide in all age groups [1]. No directed disease prevention and control strategies are approved to date and there is an urgent need to develop norovirus antivirals and vaccines. Current norovirus vaccine development is focused on recombinant expression of viral capsid protein that results in the generation of virus-like particles (VLPs), which morphologically and antigenically resemble native virions [1]. Development of human norovirus antivirals is in its infancy because of the lack of a cell culture system and an orally susceptible small animal model [2]. Early stage anti-norovirus drug development to date has relied largely on recombinantly expressed human norovirus proteins, a cell line stably expressing the type species Norwalk virus non-structural proteins (i.e., replicon-expressing cells), and the study of related murine norovirus with its cell culture system and mouse infection model [2]. In light of the difficulties in developing a reproducible human norovirus cell culture system [2], improved methods to study these viruses are needed to increase our understanding of basic processes in virus–host interactions.

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Reverse genetics is a powerful tool to generate genetically modified viruses *de novo* from cloned cDNA. The ability to manipulate the viral genome and study its effects on viral replication and pathogenesis has greatly advanced our knowledge of many RNA virus families [3]. Single-stranded, positive-sense RNA viruses are particularly suitable for reverse genetics because their RNA genome is infectious in permissive cells. However, unlike other positive-sense RNA viruses, which use a capped 5' end or an internal ribosome entry site (IRES) for efficient protein synthesis, caliciviruses require a small viral protein called VPg (viral protein, genome-linked) covalently linked to 5' end of the viral genome to prime protein synthesis [1]. This represents a challenge for the development of calicivirus reverse genetics systems because VPg is co-transcriptionally linked to the viral genome by the viral replication complex and therefore difficult to generate. Hence, the development of the feline calicivirus (FCV) reverse genetics system showing that capped genomic RNA is infectious [4] represented a significant milestone in the field. Since then, multiple reverse genetics systems for porcine enteric calicivirus, rabbit hemorrhagic disease virus, Tulane calicivirus, and murine norovirus have been developed that use *in vitro* transcribed (IVT) and capped RNA [3]. Alternative approaches of reverse genetics systems for caliciviruses that bypass the need for capped IVT-RNA have also been successful. These systems generate the infectious RNA inside cells from plasmids or T7 RNA polymerase expressing helper viruses such as vaccinia virus or fowlpox virus, whereby capping is carried out by enzymes present in the helper virus [3, 5]. In another approach, the need for a helper virus was eliminated by exchanging the T7 RNA promoter with a eukaryotic polymerase II promoter. These mRNA-like genomes are then post-transcriptionally modified by the addition of a 5' cap and 3' polyadenylation, although they are also at risk of being spliced [5]. A major bottleneck of all calicivirus reverse genetics systems is the low efficacy of initiating viral replication from capped viral RNA, significantly reducing the yields recovered compared to VPg-linked RNA. Plasmid-based approaches using a polymerase II promoter to drive murine norovirus recovery yield approximately 5×10^3 PFU/ml, which is ~10-fold less than helper-virus based systems. The most advanced reverse genetics system for murine norovirus uses capped IVT-RNA transfected into the susceptible cell line RAW 264.7 [6]. Transfection of capped IVT-RNA recovers $>4 \times 10^5$ TCID₅₀ infectious viruses from a 35 mm dish, but still falls short of the 3.4×10^7 TCID₅₀ infectious viruses recovered from VPg-linked viral RNA.

Similar plasmid-based approaches have also been applied towards the development of human norovirus reverse genetics systems, although the absence of an infectious cell culture system limits virus recovery to progeny produced after a single round of infection. The first human norovirus reverse genetics system was an adaptation of the helper virus-dependent approach successfully applied for FCV [4]. Norwalk virus GI.1 RNA was generated by transfecting a plasmid containing the complete genomic cDNA under the control of a T7 promoter into 293T cells infected with vaccinia virus MVA expressing bacteriophage T7 polymerase [7]. A year later, the same helper virus-dependent system was employed to generate genome-containing particles from a GII.3 human norovirus (U201 strain) [8]. In the present study [9], Katayama and colleagues improve on their GII.3-based system to generate a helper-free, single plasmid reverse genetics system using a strong eukaryotic polymerase II promoter from the transcription factor EF-1 α . Plasmid containing the GII.3 cDNA under the

control of the EF-1 α promoter was transfected into multiple mammalian cell lines (293T, COS7, Huh 7, and Caco-2). The polyprotein precursor was expressed and proteolytically processed into the six non-structural proteins in COS7 cells, and genomic and subgenomic RNAs were detected. Progeny virions produced from transfected COS7 cells were purified and visualized by electron microscopy and contained genomic RNA linked to VPg. Particles were ~40 nm in diameter and exhibited the same density in isopycnic cesium chloride gradients as virus isolated from a patient's stool. Viral RNA recovery was most efficient in 293T and COS7 cells, which have the ability to replicate plasmids containing the SV40 origin of replication, raising the possibility that large T antigen amplification of the plasmid may increase virus yields. Next, the system was used to generate a recombinant genome containing the GFP gene embedded in the open reading frame 1 (ORF1) between the gene encoding the NTPase (NS3) and 3A-like (NS4) proteins. This 'tagged' genome was packaged and RNA extracted from purified particles was infectious after re-transfection into COS7 cells. Additional constructs placing the GFP and luciferase genes into the capsid gene were also generated, representing the first helper-free, transient replicons. These replicons may aid in mechanistic studies of viral replication and antiviral drug screening in the future. Furthermore, viral RNA was also recovered for other human norovirus genotypes (a GII.P4-GII.3 chimeric virus TCH04-577 strain, a GII.4 Saga1 strain, and the GI.1 NV68 strain). While the absence of a human norovirus cell culture system prevents directly testing the infectivity of these particles, a proof-of-principle study demonstrated that murine norovirus (strain S7) particles recovered with the system are infectious in murine macrophage RAW 264.7 cells. These data demonstrate that this reverse genetics system is capable of generating infectious progeny virus. Demonstration that the system can be applied to human and murine noroviruses also suggests it may provide a universal approach for all caliciviruses enabling mechanistic studies in the future.

The current study also leaves some unresolved issues, including the robustness of the system. Transfection of 1.2 mg of DNA into ten 225 cm flasks was required to recover virions, making this system inefficient, and care needs to be taken to exclude possible DNA contaminations from downstream analysis. Furthermore, the very low recovery of infectious murine norovirus (3.4×10^2 TCID₅₀ in COS7 and 2×10^3 TCID₅₀ in 293T per 35 mm well), falls short compared to previously described IVT-RNA based approaches. Quantification of progeny virus release by semi-quantitative, nested reverse transcription-polymerase chain reaction (RT-PCR) and comparison of gel-band intensities to a heterologous plasmid standard makes interpretation difficult. While noroviruses typically have a short 5' untranslated region (UTR), attaching 107 nucleotides from EF-1 α to the 5' end of the viral genome was tolerated and repaired during replication by an unknown mechanism. However, the effect of this elongated 5' UTR on the efficiency of virion recovery remains unknown. Another unexplained finding was the detection of the GII.3 capsid protein only by immunofluorescence but not by Western blot 24 hours post-transfection (hpt), although the antibody is capable of recognizing denatured capsid. Future studies will undoubtedly address these and other open questions.

In conclusion, the authors presented an improved, single DNA plasmid-based reverse genetics system that allows the recovery of genome-containing human norovirus particles. The demonstration that similarly generated murine norovirus particles are infectious [9] and

that EF-1 α -driven transcription leads to the recovery of infectious feline calicivirus [10] suggests that this system can be broadly used for the recovery of infectious particles for caliciviruses. However, conclusive evidence that infectious particles are produced in case of human norovirus will only come from testing recovered progeny virus in susceptible cells or organisms. This search for susceptible cells has just been made easier by the generation of tagged virions described in the current study. Thus, this novel reverse genetics system not only represents another tool to study human norovirus replication, but may also bring us closer towards the development of a long awaited human norovirus cell culture system.

References

1. Green, KY. Caliciviridae: The Noroviruses. In: Knipe, DM.; Howley, PM.; Cohen, JI.; Griffin, DI.; Lamb, RA.; Martin, MA.; Racaniello, VR.; Roizman, B., editors. *Fields Virology*. Lippincott: Williams & Wilkins; 2013. p. 582-608.
2. Karst SM, et al. Advances in norovirus biology. *Cell Host Microbe*. 2014; 15:668–680. [PubMed: 24922570]
3. Goodfellow, IG. Calicivirus Reverse Genetics. In: Bridgen, A., editor. *Reverse Genetics of RNA Viruses: Applications and Perspectives*. John Wiley & Sons Ltd; 2013. p. 82-98.
4. Sosnovtsev S, Green KY. RNA transcripts derived from a cloned full-length copy of the feline calicivirus genome do not require VpG for infectivity. *Virology*. 1995; 210:383–390. [PubMed: 7618275]
5. Ward VK, et al. Recovery of infectious murine norovirus using pol II-driven expression of full-length cDNA. *Proc Natl Acad Sci U S A*. 2007; 104:11050–11055. [PubMed: 17581883]
6. Yunus MA, et al. Development of an optimized RNA-based murine norovirus reverse genetics system. *J Virol Methods*. 2010; 169:112–118. [PubMed: 20637238]
7. Asanaka M, et al. Replication and packaging of Norwalk virus RNA in cultured mammalian cells. *Proc Natl Acad Sci U S A*. 2005; 102:10327–10332. [PubMed: 16002473]
8. Katayama K, et al. Investigation of norovirus replication in a human cell line. *Arch Virol*. 2006; 151:1291–1308. [PubMed: 16502284]
9. Katayama K, et al. Plasmid-based human norovirus reverse genetics system produces reporter-tagged progeny virus containing infectious genomic RNA. *Proc Natl Acad Sci U S A*. 2014; 111:E4043–E4052. [PubMed: 25192933]
10. Oka T, et al. Development of a novel single step reverse genetics system for feline calicivirus. *J Virol Methods*. 2014; 207:178–181. [PubMed: 25025816]

Figure 1.

Illustrated comparison of previous and newly described GII.3-based reverse genetics systems. (A) Shows the set-up of the previously reported GII.3-based system [8]. In this approach two cDNA constructs under the control of a T7 promoter, one containing the complete U201 genome and a second containing the ORF-2 and ORF-3 subgenomic region were transfected to recover particles from MVA-T7 infected 293T cells. Panels (B) – (D) show the newly developed, single plasmid, helper virus-free approach [9]. (B) Full-length GII.3 is recovered from the transfecting plasmid containing GII.3 cDNA under the control of the EF-1α promoter. Genome-containing particles are recovered and genomic RNA (gRNA) extracted from purified particles is shown to replicate upon re-transfection into COS7 cells. (C) Depicts the same approach using a GFP-tagged genome and (D) the recovery of MNV (S7 strain) progeny virions from transfected COS7 and 293T cells.