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Genetics and reverse genetics of rotavirus

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Rotavirus is a member of the family *Reoviridae*, which have genomes consisting of 10–12 double-stranded RNA segments. The functions of proteins encoded by each segment of the rotavirus genome have been studied extensively by several methods including reassortants, temperature-sensitive mutants, isolates with rearranged RNA segments, RNAi analysis, and other procedures. However, as found for most RNA viruses, the technique of reverse genetics is required for precise genotype/phenotype correlation, for the analysis of the role of specific mutation in replication process and pathogenesis, and for the development of vectors and vaccines. In 2006, we presented the first description of a reverse genetics system for rotavirus, although a helper virus and a selection system are required. Since then, two other approaches have been reported for rotavirus reverse genetics, both requiring the presence of a helper virus. A tractable, helper virus-free reverse genetics system for rotavirus has not been developed so far, in contrast to the recent developments of plasmid only-based reverse genetics systems for other members of the *Reoviridae*.

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Introduction

Extensive studies on rotavirus genetics have been carried out since the discovery of human rotavirus in 1973 [1,2]. Progress in methodology enhanced the genetic studies. This has included the cultivation of human rotavirus in cell culture, preparation of reassortants and *temperature (ts)* mutants, *in vitro* transcription involving double-layered particles (DLPs), RNA-RNA hybridization, recombinant baculovirus expression systems, the application of specific RNAi, and other procedures. When a report on *in vitro* RNA replication of rotavirus involving open cores appeared in 1994 [3], most rotavirus researchers thought that the development of rotavirus reverse genetics would follow soon. However, it took more than 10 years for a report on the successful development of such a system. In 2006, we succeeded in the establishment of a reverse genetic system of rotavirus, although it required a helper virus and a strong selection system [4••,5]. This success for rotavirus triggered the development of reverse genetics for other members of the *Reoviridae*. In 2007, a plasmid only-based reverse genetics was developed for orthoreovirus [6••], the efficiency of which was later improved further [7,8]. By applying this reverse genetics system to orthoreovirus, various new contributions were made to elucidate different steps of the viral replication cycle [9,10]. Furthermore, for bluetongue virus and African horse sickness virus of the genus *Orbivirus* in the family *Reoviridae*, the transfection of *in vitro* transcribed RNA from cDNA or of mRNA prepared *in vitro* from single-shelled core particles into cultured cells yielded infectious viruses [11,12••,13]. For rotavirus reverse genetics, two other methods were developed in 2010 [14•,15••]. However, all systems including the one developed by our group [4••] require a recombinant vaccinia virus expressing T7 RNA polymerase and particular ways to exert selection pressure. So far, a generally applicable plasmid only-based reverse genetics of rotavirus has not been developed for rotavirus, despite extensive efforts of many research groups. This is a mystery. Why is it so difficult to develop a tractable, true reverse genetics system for rotavirus?

In this review, after touching upon the classical genetics of rotavirus, we summarize the reverse genetics for multi-segmented double-strandes (ds)RNA viruses such as members of the *Birnaviridae* (2 segments), *Cystoviridae* (3 segments) and the viruses of the *Reoviridae*. Then, we describe the development of reverse genetics for rotavirus in different systems and provide a few examples of its application by site-directed mutagenesis of the rotavirus VP4 gene. Finally, we envisage the future developments of rotavirus reverse genetics.

Genetics of rotavirus

Rotavirus has a genome consisting of 11 dsRNA segments, which encode six structural proteins and six non-structural proteins. The function of each protein has been elucidated by various means such as preparation of reassortants, viruses with rearranged gene segment, and *ts* mutants, and knockdown of a given viral protein using siRNAs or intrabodies.

Reassortants

When cells are co-infected with two different but compatible rotaviruses (of the same group/species), a high

percentage of progeny viruses contain novel assortments of gene segments (reassortants). Studies involving a number of reassortants derived from two strains with different phenotypes contributed very effectively to mapping of the phenotypes to specific viral segmental gene products [16,17,18•]. For example, VP4 and NSP1 were found to determine the distinctive replication capacities in the mouse biliary tract [19]. VP3, VP4, VP7, and NSP4 were found to be associated with virulence and host range restriction [20,21].

Rearrangement

Rearrangement denotes alterations of considerable portions of the sequence within single genome segments, sometimes in the form of deletions, and often as partial duplication. Several variants with rearranged segments and altered phenotypes have been isolated [22]. Since the virus with a rearranged gene 5 (encoding NSP1) with an abrogated open reading frame of only 40 amino acids grows well in cell cultures and induces diarrhea in mice, NSP1 was found not to be essential for virus growth *in vitro* and *in vivo* [23].

Temperature-sensitive mutants

Various *ts* mutants have been prepared by treatment of rotavirus with mutagenic chemicals. Rotavirus *ts* mutant groups (A to G, J and K) have now been mapped to 9 of the 11 rotavirus genome segments [18•], and the amino acid mutations on each protein have been identified. The two reassortment groups H and I will be assigned to the remaining segments encoding NSP3 and NSP4 in the near future. Greenberg *et al.* [24] first identified VP4 being the hemagglutinin and the virus protein that is responsible for noncultivatability of human rotavirus by using *ts* mutants.

Intrabodies

Intracellular antibodies or intrabodies have great potential as protein knockout strategies for intracellular antigens. Expression of intracellular antibody to NSP5 was found to largely reduce the assembly of viroplasms, cellular cytopathic effect and the titer of infectious viral progeny [25].

siRNAs

The highly specific and efficient inhibition of viral gene expression by siRNAs offers the potential to elucidate the function of a given viral protein, first analyzed for VP4 [26]. By RNAi analysis NSP5 was found to have a pleiotropic effect on virus replication [27]. The siRNA approach showed that NSP5 is essential for the assembly of viroplasms that are associated with the utilization of lipid droplet components for rotavirus replication [28]. Silencing of NSP4 was found to have unexpected global consequences for virus replication as well as protein localization in infected cells [29,30]. NSP4 knockdown dramatically increased levels of viral transcription and inhibited formation of packaged virus particles [29]. By NSP4 silencing, it was confirmed that NSP4 is the main protein responsible for the changes in Ca^{2+} homeostasis in cultured cells [31]. In other studies, silencing NSP4 downregulated VP7 and VP4, and resulted in a decreased incidence of biliary atresia [32].

Thus, the major functions of each protein were analyzed by the various methods described above. However, analyses involving forward genetics have limitations such as the inability to identify the precise functional region in the genome by using virions with genomes that have arbitrary substitutions, insertions, or deletions.

Reverse genetics is a powerful technique for studying precisely the function of each segment and its protein product in an otherwise unchanged genetic background and for identifying the regions of the segments important for RNA transcription, replication and packaging.

Reverse genetics of dsRNA viruses with a segmented genome

There are many viruses with multi-segmented dsRNA as a genome. Since the packaging mechanism of multiple dsRNA segments is unknown, genetic modification has been notoriously difficult. Since the success for bacteriophages ϕ6 and ϕ8 with a genome of three dsRNA segments [33–40], however, much progress has been made on the reverse genetics for multi-segmented dsRNA viruses.

Bacteriophages ϕ6 and ϕ8

Bacteriophages ϕ6 and ϕ8 are members of the family *Cystoviridae*, which have three dsRNA segments: L, M, and S. In 1990, Olkkonen *et al.* [33] developed a reverse genetics system. They prepared cDNA-derived single-stranded (ss) RNA of M segment by using a plasmid encoding M segment under the control of T7 RNA polymerase promoter. After mixing with natural L and S ssRNAs, the cDNA-derived M ssRNA was subjected to *in vitro* packaging-replication system that has been developed by Gottlieb *et al.* [34]. After coating on outer shell protein P8, the nucleocapsids were infected onto *Pseudomonas phaseolicola* spheroplasts, yielding infectious bacteriophage ϕ6. Onodera *et al.* [35] modified this first system. They prepared a replication-incompetent ϕ6 derivative containing the L segment with a deletion by the *in vitro* packaging-replication system. The *P. phaseolicola* expressing the intact ϕ6 L segment ssRNA transcribed by SP6 RNA polymerase was then infected with the replication-incompetent ϕ6 derivative. As a result, they could obtain the reverted infectious ϕ6 that possess the functional cDNA-derived L segment. Later, Onodera *et al.* could prepare novel engineered viable viruses that possess genomic segments joined together so that the number of the segment can be only one or two [36].

Finally, it is now possible to produce infectious virions by electroporating three plasmids encoding L, M and S segment, respectively, under T7 or SP6 RNA polymerase promoter into the spheroplasts expressing T7 or SP6 RNA polymerase [40].

Birnavirus

Infectious bursal disease virus (IBDV) and infectious pancreatic necrosis virus (IPNV) are members of the *Birnaviridae*, and have a genome consisting of two dsRNA segments. By transfection of mRNA prepared *in vitro* from cores or ssRNA prepared *in vitro* from cDNA clones into Vero cells, a cell line from African green monkey kidney epithelial cells, infectious IBDV and IPNV were recovered [41,42]. Later, QM5 cells were infected with chicken pox virus expressing T7 RNA polymerase, and two transcription plasmids encoding each of the two RNA segments of IBDV were transfected, and an infectious IBDV derived entirely from cDNA was recovered [43].

Orthoreovirus

The basis for reverse genetics for ortho reovirus with a genome of 10 dsRNA segments was the discovery in 1990 that ortho reovirus RNA is infectious [44]. This system is unique and complicated. A lysate of rabbit reticulocyte in which ssRNA has been translated, and ssRNA and/or dsRNA from serotype 3 ortho reovirus (ST3) were transfected into L929 cells, and then the cells were infected with serotype 2 ortho reovirus (ST2). On day 5 after the infection, plaques of ST3 virus were recovered, since ST2 virus needs at least 12 days for plaque formation. By this and its modified method, packaging signals were identified [45] and a foreign CAT gene was incorporated into the genome [46]. However, these methods are delicate and tricky to handle, and have not been reproduced in other laboratories. In 2007, a plasmid only-based reverse genetics system not requiring a helper virus nor a selection system, was developed for orthoreovirus [6••]. In this system, transcription of each gene segment located downstream of the T7 RNA polymerase promoter is driven by T7 RNA polymerase, supplied transiently by recombinant vaccinia virus (rDIs-T7pol) or by cells that constitutively express the enzyme [7]. This excellent reverse genetics system has been developed for two prototype orthoreovirus strains, type 1 Lang and type 3 Dearing. For the first generation rescue system, each cDNA for 10 genes was inserted into a separate plasmid. In the second-generation system, the efficiency of virus recovery was enhanced by engineering the cDNAs of multiple segments into single plasmids to reduce the number of plasmids to be transfected from 10 to 4 [7]. Furthermore, the use of baby hamster kidney cells that express T7 RNA polymerase (BHK-T7) eliminated potential biosafety concerns associated with the use of recombinant vaccinia virus [7,8]. The orthoreovirus reverse genetics has been applied to the analysis of viral protein functions [9,10]. Specific mutations in the attachment protein σ1 have been introduced by reverse genetics [9]. Since orthoreoviruses induce cell death and apoptosis in tumor cells, but not in healthy non-transformed cells [47], reverse genetics for orthoreovirus may be useful for generating new orthoreovirus-derived oncolytic agents and vaccines. Furthermore, it is possible to introduce different exogenous peptides, up to 40 amino acids long, at the carboxyl-terminal end of the σ1 outer capsid protein [48].

Orbivirus

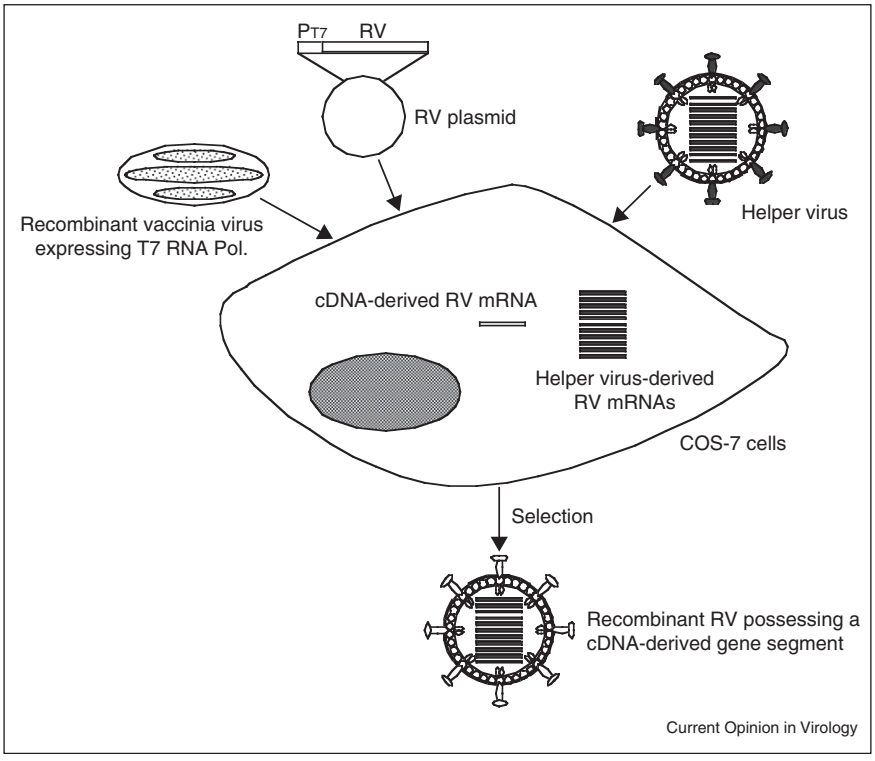
For bluetongue virus belonging to the *Orbivirus* genus of the *Reoviridae*, it became possible to recover infectious bluetongue virus by transfection of permissive cells with the complete set of 10 purified viral mRNAs derived *in vitro* from transcribing cores [11] or *in vitro* synthesized T7 transcripts using cDNA templates [12••]. Also, for African horse sickness virus, another member of the *Orbivirus* genus, a similar system has been developed [13]. Recently, a cell-free system for reconstituting functional subcore and core structures with proteins and ssRNAs was developed for bluetongue virus [49].

Reverse genetics for rotavirus

Use of neutralizing antibodies

A major breakthrough in genetic engineering of rotaviruses occurred in 2006 when we pioneered a reverse genetics system that enables the generation of infectious rotaviruses containing a cDNA-derived gene segment [4••]. This rotavirus system is based on helper virus-driven reverse genetics (Figure 1), which was originally developed for influenza viruses [50–52]. This system is based on the cloned full-length VP4 gene of simian rotavirus strain SA11 (G3P[2]), flanked by the T7 RNA polymerase promoter and hepatitis delta virus (HDV) ribozyme, and followed by the T7 RNA polymerase terminator (Figure 2A). The resulting plasmid was transfected into COS-7 cells that had been infected with recombinant vaccinia virus (rDIs-T7pol) to provide T7 RNA polymerase. Each of the authentic 5′ cap and 3′ polyA tail-lacking structures of rotavirus mRNA ends had to be obtained with vaccinia virus-encoded capping activity and HDV ribozyme sequences, respectively. The transfected cells were superinfected with human rotavirus strain KU (G1P[8]) as a helper virus. Recombinant rotavirus possessing a cDNA-derived SA11 VP4 gene (P[2]) segment was rescued by P[8] specific neutralizing antibody selection against the KU helper virus. In addition to the recombinant virus having the authentic VP4 gene, three more recombinant rotaviruses, into which silent mutation(s) had been introduced as gene markers in the VP4 genome, were soon generated using this method (Figure 2A). Hence, the feasibility of this rotavirus reverse genetics system was confirmed. The keys for the development of the rotavirus reverse genetics system are the use of specific anti-VP4 neutralizing monoclonal antibodies [53] and the use of recombinant vaccinia virus expressing T7 RNA polymerase (rDIs-T7pol) that is non-pathogenic to mammalian cells [54•].

Figure 1

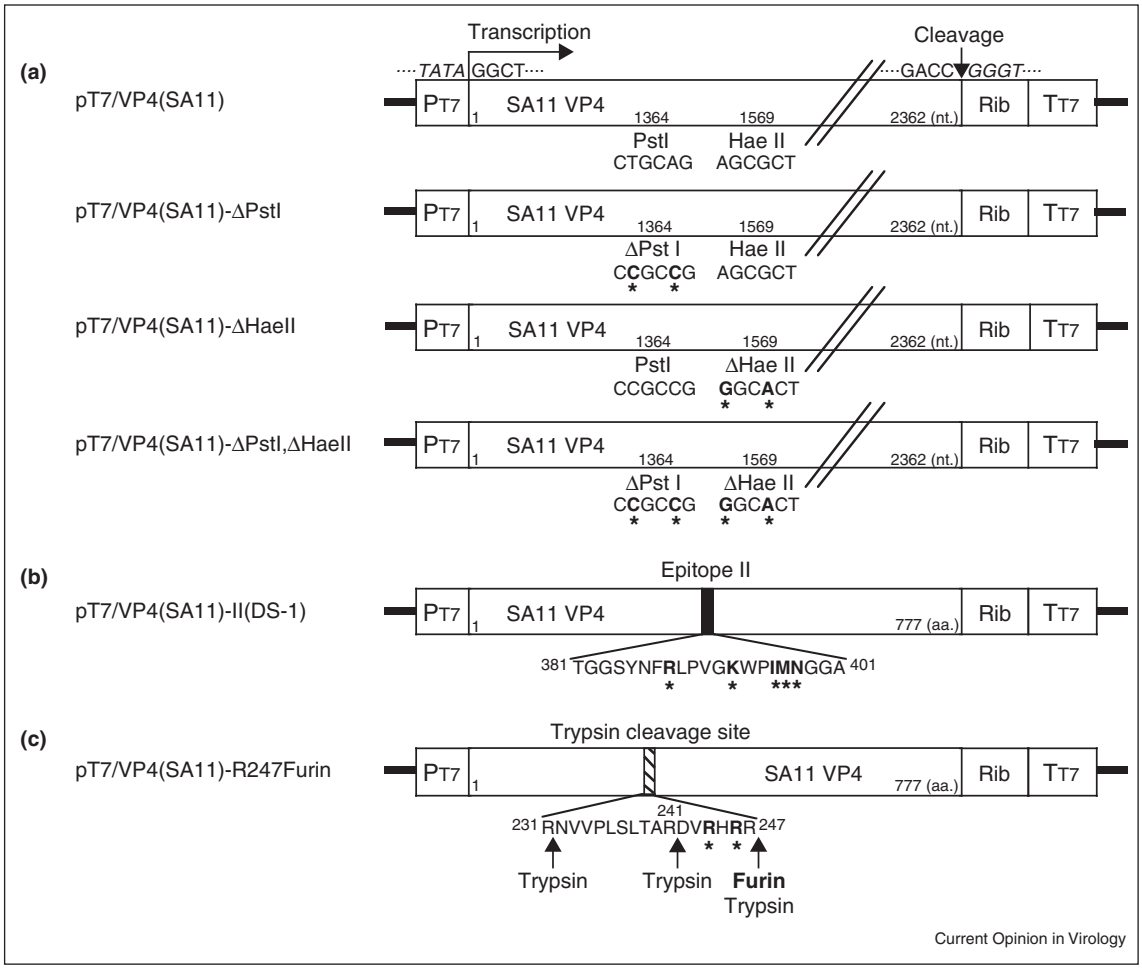


Reverse genetics system for rotavirus (RV). A plasmid containing a RV cDNA gene insert is transfected into COS-7 cells that have been infected with a recombinant vaccinia virus expressing T7 RNA polymerase. Cells are then infected with a helper virus. Intracellular transcription by T7 RNA polymerase yields artificial RV mRNA, which is packaged into progeny virions. The generated recombinant RV is rescued from the background of helper viruses by applying strong selection systems.

With this method, recombinant rotaviruses with artificial amino acid substitutions were generated. Site-specific mutations were introduced into one of the three cross-reactive neutralization epitopes of VP4, which resulted in the engineering of recombinant rotavirus expressing chimeric VP4 on its surface [55••]. The rescued virus contains SA11 (G3P[2])-based VP4, in which a cross-reactive neutralization epitope on VP5* was replaced by the corresponding sequence of a different P-type DS-1 (human rotavirus strain, G2P[4]) (Figure 2B). Serological analyses with a panel of anti-VP4 and anti-VP7-neutralizing monoclonal antibodies revealed that the engineered virus carried a novel antigenic mosaic of cross-reactive neutralization epitopes on its VP4 surface. Clearly, this approach to construct chimeric rotaviruses will potentially lead to a new generation of effective vaccine candidates against rotavirus disease.

The spike proteins of many enveloped viruses are initially synthesized as inactive precursors. Therefore, proteolytic cleavage of precursor spike proteins at mono-basic or multi-basic cleavage site(s) by cellular proteases is absolutely required to convert them into an active state and to render the virions infectious. The mono-basic cleavage sites and multi-basic cleavage sites are readily cleaved by exogenous trypsin-like proteases and endogenous furin-like proteases, respectively. VP4, the spike protein of non-enveloped rotavirus, resembles the precursor spike proteins of enveloped viruses because they exhibit substantial structural and functional similarities. VP4, as an inactive precursor, is cleaved at mono-basic cleavage sites by trypsin into VP5* and VP8* as active states, resulting in activation of rotavirus infectivity [56]. To examine the possibility that modification of the VP4 trypsin cleavage site to a furin-sensitive state would allow engineering of a viable rotavirus that can perform multicycle replication without trypsin, we generated and characterized recombinant rotavirus expressing a mutant VP4 that can be cleaved by furin-like proteases as well as trypsin [57] (Figure 2C). Unexpectedly, this VP4 mutant could not undergo multicycle replication without an exogenous protease, although the mutant VP4s on virions were efficiently cleaved by furin-like proteases. Since nascent viruses containing already cleaved VP4 were significantly constrained within the cells, it was suggested that intracellular cleavage of VP4 by furin may be disadvantageous for rotavirus infectivity, possibly owing to an inefficient virus release process. This reverse genetics approach may become valuable for the understanding of the molecular basis

Figure 2



Schematic representation of the SA11 virus-based transcription plasmids encoding the full-length VP4 gene. **(A)** The wild-type and mutated VP4 genes of SA11 were cloned between the T7 RNA polymerase and HDV ribozyme, followed by the T7 RNA polymerase terminator. The numbers indicate the nucleotide positions in the SA11 VP4 sequence. **(B and C)** Manipulations of the VP4 gene by means of amino acid mutations (positions are indicated by bold letters and asterisks below the sequences) were carried out in pT7/VP4(SA11). The mutant plasmid pT7/VP4(SA11)-II(DS-1) contains five amino acid mutations within the epitope II sequence **(B)**. The mutant plasmid pT7/VP4(SA11)-R247Furin possesses two amino acid mutations within the trypsin cleavage site that create a furin cleavage site at the R247 position **(C)**. The numbers indicate the amino acid positions in the SA11 VP4 sequence. PT7, Rib, and TT7 denote the T7 RNA polymerase promoter, HDV ribozyme, and T7 RNA polymerase terminator, respectively.

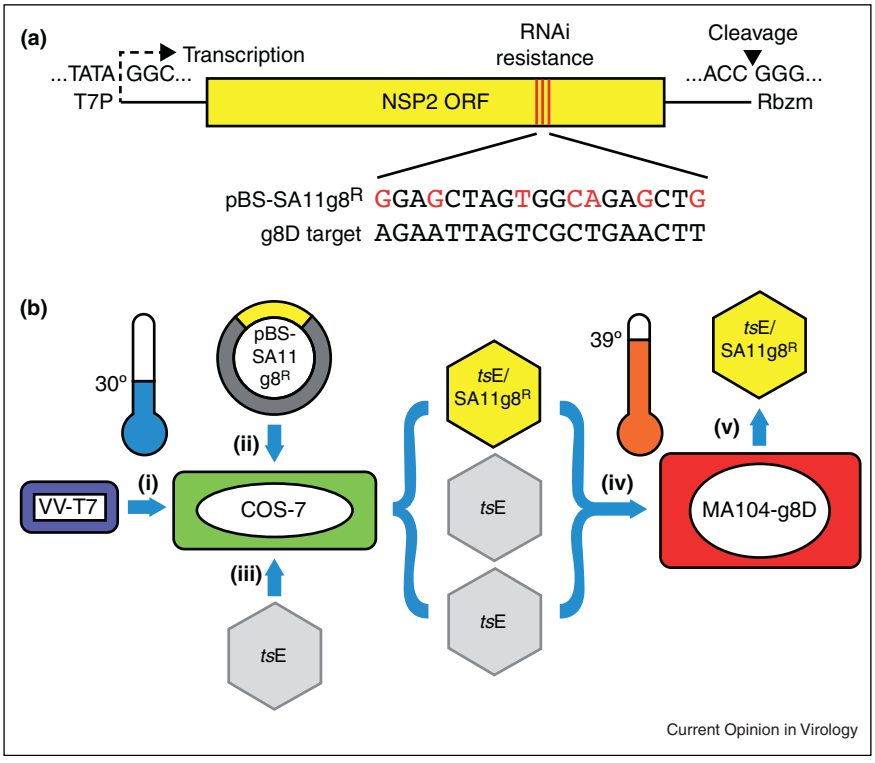
of the significance of VP4 and VP7 for rotavirus replication and pathogenesis.

VP4 possesses three conserved mono-basic residues at its trypsin cleavage site (R231, R241 and R247) (Figure 2C). Although only R247 is assumed to be required for the activation of infectivity with conventional techniques, the strict conservation of the three residues suggests that the presence of these residues may play an important role during infection. This possibility was evaluated by generating recombinant rotaviruses with trypsin cleavage site mutations using reverse genetics, and the role of each arginine residue in rotavirus infectivity will be demonstrated in the near future [Komoto and Taniguchi, unpublished data].

The present method is applied only for the VP4 gene, but can be extended to the VP7 gene. The SA11-L2 clone has little dependency on trypsin for its growth [58]. On transfection of an artificial SA11-L2 VP7 gene in the presence of neutralizing monoclonal antibodies specific to VP7 (G1-specific) as a selection pressure and the use of a KU-SA11 reassortant whose VP4 gene only is derived from SA11 as a helper virus, recombinant viruses with the VP4 and VP7 genes, both of which are derived from SA11-L2, independent of trypsin would be isolated.

Furthermore, if a marker protein such as GFP can be expressed in this system, the use of the IRES sequence to prepare a bicistronic segment (GFP and NSP protein) will also have the potential as a modified reverse genetics

Figure 3



Procedure for generation and recovery of gene 8 recombinant virus using dual selection [15••]. **(A)** Schematic representation of the SA11 virus-based transcription plasmids encoding the full-length gene 8. **(B)** Procedure for reverse genetics under dual selection. COS-7 cells are infected with rDIs-T7pol (VV-T7) **(i)**, and transfected with the recombinant gene 8 plasmid (pBS-SA11g8^R) **(ii)** before infection with the tsE helper virus at 30°C **(iii)**. The recovered virus stock may have the tsE/SA11g8^R virus as well as tsE helper virus. Passage in MA104-g8D at 39°C **(iv)** permits efficient isolation of the tsE/SA11 g8^R virus **(v)**.
From [15••], with permission of the authors and the publisher.

technique permitting the selection of fluorescing cells by FACS or any other method, since rotavirus particles have the ability to pack the extra nucleotides of 1900–2400 nucleotides [[59], Chen *et al.*, unpublished data]. In particular, NSP1 is found to be non-essential for rotavirus growth in cultured cells or in mice [23], and the NSP1-coding region might be replaced by the GFP gene.

Use of *ts* mutant

Trask *et al.* [15••] developed reverse genetics protocol in which a mutant SA11 rotavirus encoding a *ts* defect in the NSP2 protein and RNAi-mediated degradation of NSP2 mRNA are employed. They prepared a plasmid pBS-SA11g8 that has an SA11 NSP2-coding gene 8 cDNA with a T7 RNA polymerase promoter sequence at the 5′ end and a modified HDV ribozyme and T7 RNA polymerase terminator at the 3′ end (Figure 3A). Seven mutations were introduced into the SA11g8 cDNA in the region targeted by the g8D siRNA to generate plasmid pBS-SA11g8^R. COS-7 cells were infected with the T7 RNA polymerase-expressing rDIs-T7pol and then transfected with the pBS-SA11g8^R plasmid. The cells were then infected with rotavirus *ts*E as a helper virus and incubated at 30°C. Virus in the cell lysates was passaged at 39°C in MA104 cells that express g8D siRNA to select for progeny virus with the recombinant gene 8 (Figure 3B). In this system, the *ts* mutant is the much more critical selection mechanism than the use of siRNA. The utility of this reverse genetics system was evaluated by obtaining recombinants expressing chimeric NSP2 proteins.

This dual selection with *ts* mutants and RNAi can theoretically be extended to other genes, since rotavirus *ts* mutation groups have been mapped to 9 segments [18•], except for gene segments encoding NSP3 and NSP4. For this approach to be successful, the *ts* mutants must be very tight.

Use of rearranged gene

Troupin *et al.* [14•] reported a reverse genetics system for rotavirus based on the preferential packaging of rearranged RNA segments. They introduced an *in vitro*-engineered gene 7 with rearrangement encoding a modified NSP3 protein into an infectious rotavirus. In this system, they also used COS-7 cells infected with rDIs-T7pol for providing T7 RNA polymerase and a bovine

rotavirus RF as a helper virus, but no additional selective pressure. This system can theoretically be applied to other genes (genes 5–11) for which rearrangements have been described. The preferential packaging of the rearranged RNA segments might be caused by the duplication of packaging signals or secondary structures in rearranged segments [60].

Plasmid only-based

Extensive studies aiming at establishing a plasmid only-based rotavirus reverse genetics system have been performed without success so far. Why is it so difficult to develop plasmid only-based rotavirus reverse genetics in contrast to the successes with orthoreoviruses and orbiviruses? In our laboratory we could readily confirm the excellent efficiency of the orthoreovirus reverse genetics system [6••] by using a set of 10 plasmids, each containing the cDNA of one segment, and L929 cells provided by Dr. Dermody. As a result, we recognized that there are no problems with reagents and techniques used for rotavirus in our laboratory. Rotaviruses have one more RNA segment than orthoreovirus. Rotavirus infection depends on the presence of trypsin more than orthoreovirus. The ratio of the number of virus particles per infectious unit is much lower in rotavirus [61]. It will be the problem of the efficiency of the recovery of infectious rotavirus particle. There might be some modifications necessary for the success of plasmid only-based rotavirus reverse genetics such as preparation of plasmids having multiple gene inserts in order to reduce the number of plasmids for transfection, use of rotavirus strain whose dependency on trypsin is low, use of cells that express some of the rotavirus proteins and T7 RNA polymerase, or other procedures. We have examined some of them. We employed a set of 11 transcription plasmids encoding each gene segment from some animal and human rotavirus strains. The use of various cell-lines susceptible (COS-7, 293T, MA104, CV-1, HT-29, MDCK and Vero cells) and refractory (BHK, CHO and L929 cells) to rotavirus infection were examined. We used the cells that constitutively express T7 RNA polymerase. Inclusion of eukaryotic expression plasmids encoding rotavirus proteins was also examined. However, these approaches did not lead to success so far. Further persevering in investigations will be required for the success of the plasmid only-rotavirus reverse genetics.

Conclusion

Since our early success in rotavirus reverse genetics, progress has been slow. There are three different systems for rotavirus reverse genetics so far, whose reported target gene segments are VP4, NSP2 and NSP3, respectively. Theoretically, they can be extended to more gene segments. In addition, the efficiency of obtaining infectious rotavirus with a segment having undergone site-directed mutagenesis should be improved. Ultimately, as for other members of the *Reoviridae*, plasmid only-based reverse genetics for rotavirus should be developed by extensive examinations of variables that will affect the recovery efficiency of infectious virus.

Once a plasmid only-based reverse genetics system has been developed, the procedure will help to solve problems such as the precise mechanism of virus entry into cells and virus export from cells, control mechanism of packaging of 11 RNA segments into particles, mechanism of protein and vesicle trafficking, pathway of developing viremia/antigenemia, identification of the region responsible for pathogenesis and attenuation, and many others.

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