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REVIEW

Genetic engineering of rotaviruses by reverse genetics

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ABSTRACT

The rotavirus genome is composed of 11 gene segments of dsRNA. A recent breakthrough in the field of rotaviruses is the development of a reverse genetics system for generating recombinant rotaviruses possessing a gene segment derived from cloned cDNA. Although this approach is a helper virus-driven system that is technically limited and gives low levels of recombinant viruses, it allows alteration of the rotavirus genome, thus contributing to our understanding of these medically important viruses. So far, this approach has successfully been applied to three of the 11 viral segments in our laboratory and others, and the efficiency of recovery of recombinant viruses has been improved. However, we are still waiting for the development of a helper virus-free reverse genetics system for generating an infectious rotavirus entirely from cDNAs, as has been achieved for other members of the *Reoviridae* family.

Key words helper virus, *Reoviridae*, reverse genetics, rotavirus.

Rotavirus, a member of the family *Reoviridae*, is the leading etiological agent of severe gastroenteritis in infants and young children worldwide, being responsible for an estimated 453,000 deaths annually (1). Rotavirus is a non-enveloped virus comprising three concentric layers enclosing a genome of 11 segments of dsRNA, which encodes six structural proteins (VPs) and six non-structural proteins (NSPs) (2). Rotavirus has two outer capsid proteins, VP7 and VP4, which are involved independently in neutralization, and define the G and P genotypes, respectively.

Remarkable progress as to the understanding of various aspects of viruses has been achieved through reverse genetics technology that allows one to generate infectious viruses possessing gene(s) derived from cDNA (s). Although reverse genetics systems exist for nearly all major groups of DNA- and RNA-viruses, development of such a system for *Reoviridae* viruses is more challenging owing in part to the technical complexity of manipulation of their multi-segmented genomes (3, 4). Through the forward genetics approach and reassortant analysis, studies of rotavirus have yielded a wealth of knowledge of virus replication and pathogenesis. However, the ability to introduce specific changes into the rotavirus genome has been limited. Reverse genetics is a powerful approach both for an understanding of virus replication, and for the development of vaccines and viral vectors.

The entire genome nucleotide sequence was determined first for a rotavirus strain in 1990 (5). Since then, researchers have managed to clone the cDNAs used for sequencing and to recover infectious rotaviruses from the cloned cDNAs. When the development of an *in vitro* replication system was reported in 1994 (6), most investigators anticipated that the development of a rotavirus reverse genetics system would quickly follow. However, for a decade, no recombinant rotaviruses were rescued at all, despite intensive attempts around the world. A breakthrough in the field of rotavirus reverse genetics came in 2006, when we established the first reverse genetics system for rotaviruses, which is a partially plasmid-based system that permits replacement of a viral gene segment with the aid of a helper virus (7). This achievement opened the door to the genetic engineering of rotaviruses. Although this helper virus-driven system is

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List of Abbreviations: dsRNA, double-stranded RNA; HDV, hepatitis delta virus; iPS cells, induced pluripotent stem cells; PFU, plaque-forming unit; RdRp, RNA-dependent RNA polymerase; RNAi, RNA interference; siRNA, small interfering RNA.

technically limited and gives low levels of recombinant viruses, it allows alteration of the rotavirus genome, thus contributing to our understanding of these medically important viruses. To date, this approach has been used to generate recombinant rotaviruses with cDNA-derived VP4 (7, 9), NSP2 (10, 11), and NSP3 (12) gene segments.

In the present review, we describe the development and application of our rotavirus reverse genetics system, and its future perspectives.

PRINCIPLES OF A ROTAVIRUS REVERSE GENETICS SYSTEM

Overview of the rotavirus replication cycle

A rotavirus virion binds to specific receptors and enters target cells (13). During the internalization, the outer capsids are removed, and transcriptionally active double-layered particles are delivered into the cytoplasm (14). The RdRp complexes packaged within the viral core transcribe each of the dsRNA gene segments and release the positive-strand RNAs into the cytoplasm (15). The rotaviral positive-strand RNAs are structurally unique, being capped at the 5′ terminus but not adenylated at the 3′ terminus (16). The positive-strand RNAs serve as both mRNAs for the translation of viral proteins and templates for negative-strand RNA synthesis during genomic dsRNA replication within the new viral core (6). Viral structural and non-structural proteins form large cytoplasmic inclusions (viroplasms) where genome replication and virion assembly occur (17). The virions are released from the cells through lysis.

Development of a rotavirus reverse genetics system

Based on the rotavirus replication cycle described above, it was theoretically conceivable that a cDNA-derived positive-strand RNA corresponding to native viral mRNA introduced into rotavirus-infected cells should be recognized and replicated to yield dsRNA by means of viral RdRp complexes, yielding an infectious rotavirus containing a gene segment derived from cDNA. In 2006, we developed a rotavirus reverse genetics system that enables the generation of infectious rotaviruses possessing a cDNA-derived gene segment according to this scenario (7) (Fig. 1). Our procedure for rescuing recombinant rotaviruses is based on a helper virus-driven reverse genetics system, which was originally developed for influenza virus (eight-segmented negative-strand RNA virus) (18, 20). To engineer a transcription plasmid that would yield a rotavirus mRNA replica, we cloned the full-length VP4 gene (encoding a spike protein) of simian rotavirus strain SA11 (G3P[2]), flanked by the T7 RNA polymerase promoter and HDV ribozyme, and followed by T7 RNA polymerase terminator sequences (Fig. 2). The T7 RNA polymerase initiates transcription at a defined guanosine residue (21). The rotavirus mRNAs contain the consensus nucleotide sequence 5′-GGC(A/U)(A/U)U-3′ at their 5′ terminus.

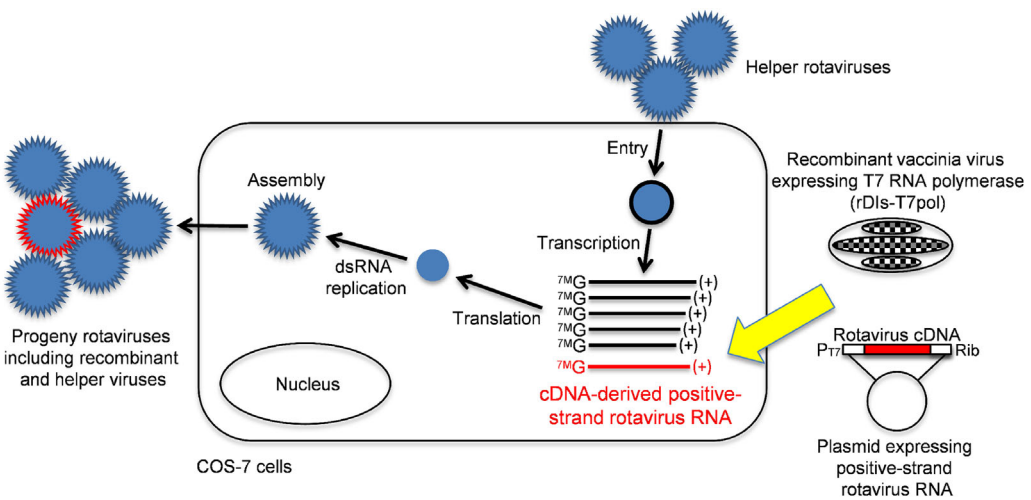

Fig. 1. Rotavirus reverse genetics system. A T7 plasmid containing a rotavirus gene segment cDNA is transfected into COS-7 cells that have been infected with recombinant vaccinia virus expressing T7 RNA polymerase (rDIs-T7pol). Transfected cells are then infected with helper rotaviruses. Intracellular transcription by T7 RNA polymerase yields a cDNA-derived positive-strand RNA corresponding to native viral mRNA, which is packaged into progeny viruses. A recombinant rotavirus possessing a cDNA-derived gene segment is rescued from the background of helper viruses by applying selection mechanisms. PT7 and Rib denote the T7 RNA polymerase promoter and HDV ribozyme, respectively.

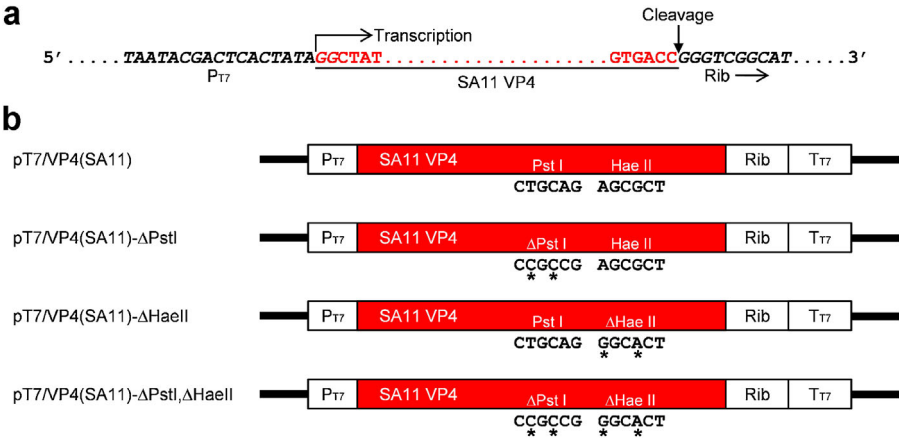

Fig. 2. Schematic representation of T7 plasmids encoding the full-length VP4 gene segment of SA11. (a) The sequences at the 5′ and 3′ termini of the VP4 gene in T7 plasmid. A nascent T7 transcript corresponding to viral mRNA contains the native 5′ terminal sequence. Self-cleavage by HDV ribozyme produces an RNA with the native 3′ terminus without the polyA-tail. (b) Cloned wild-type and mutated VP4 genes are flanked by the promoter sequence for T7 RNA polymerase (PT7) and HDV ribozyme (Rib), followed by the T7 RNA polymerase terminator (TT7). Manipulation of the VP4 gene by means of silent mutations (positions are indicated by asterisks below the sequences) was carried out in the pT7/VP4(SA11) plasmid. The mutated plasmids contain the destruction mutation(s) at a unique *Pst*I or *Hae*II site, or both, respectively.

Thus, the use of T7 RNA polymerase to catalyze the transcription of rotavirus RNA produces positive-strand RNA with a native 5′ terminal sequence. Self-cleavage of the antigenomic HDV ribozyme sequence will produce RNA with the native rotavirus 3′ terminus but without the polyA-tail (22) (Fig. 2a). The resulting T7 plasmid was transfected into simian kidney COS-7 cells that had been infected with recombinant vaccinia virus expressing T7 RNA polymerase (rDIs-T7pol; 23). The authentic 5′ cap structure of rotavirus mRNAs was to be obtained with vaccinia virus-encoded capping enzyme activity (24). Therefore, the T7 plasmid was presumed to generate a transcript corresponding to native mRNA of VP4 that serves as a template for translation and dsRNA replication in the replication cycle of rotaviruses. The transfected cells were then infected with a human rotavirus strain KU (G1P[8]) as a helper virus. By passaging the cell lysates in MA104 cells in the presence of P[8]-specific neutralizing monoclonal antibodies against the KU helper virus, a recombinant rotavirus containing a cDNA-derived SA11 VP4 segment (P[2]) was rescued. In addition to the first recombinant rotavirus possessing the authentic VP4 gene segment, three more recombinant rotaviruses, into which silent mutation(s) had been introduced as gene markers in the VP4 gene, were soon rescued with this procedure (7) (Fig. 2b). Thus, the utility of this reverse genetics system was confirmed. The key points for the development of a rotavirus reverse genetics system were the use of serotype-specific anti-VP4 neutralizing monoclonal antibodies, attenuated recombinant vaccinia virus expressing T7 RNA polymerase (rDIs-T7pol), and highly transfectable COS-7 cells.

USE OF THE ROTAVIRUS REVERSE GENETICS SYSTEM

Using the reverse genetics system thus developed, we next generated a recombinant rotavirus expressing chimeric VP4s on its surface. A previous study had revealed the presence of three cross-reactive neutralization epitopes, I–III, on VP4 molecules (25). Multiple site-directed mutations were introduced into epitope II for expression in the chimeric VP4 because it is thought to be highly immunodominant (25, 26), which resulted in the engineering of a novel recombinant rotavirus expressing chimeric VP4s on its surface (8). This was the first report of the generation of a recombinant rotavirus with artificial amino acid substitutions. The rescued virus contains SA11 (G3P[2])-based VP4, in which epitope II was replaced with the corresponding sequence of a different P genotype DS-1 (human rotavirus strain, G2P[4]) (Fig. 3a). The antigenic properties of the rescued virus were investigated by an ELISA and a plaque neutralization assay, using a panel of anti-VP4 and -VP7 neutralizing monoclonal antibodies. These serological analyses revealed that the rescued virus expresses both the DS-1- and SA11-derived epitopes on its VP4 surface; that is, this chimeric virus carries the designed antigenic mosaic of cross-reactive neutralization epitopes on its VP4 surface. Undoubtedly, this approach for preparing

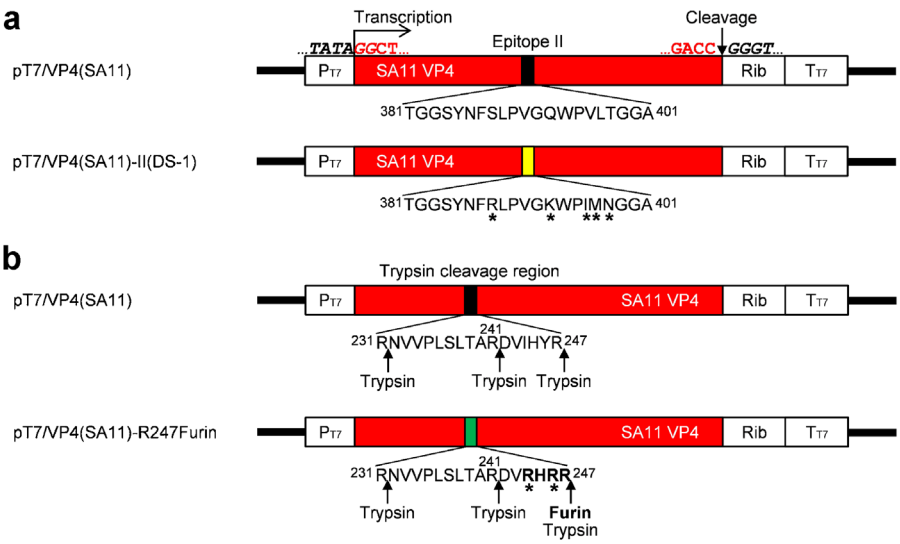

Fig. 3. Generation of recombinant rotaviruses expressing cDNA-derived VP4 with artificial amino acid substitutions. Manipulations of the VP4 gene by means of amino acid mutations (positions are marked by asterisks below the sequences) were carried out in the pT7/VP4(SA11) plasmid. (a) The mutant plasmid, pT7/VP4(SA11)-II(DS-1), possesses five amino acid mutations within the epitope II sequence. (b) The mutant plasmid pT7/VP4(SA11)-R247Furin contains two amino acid mutations within the trypsin cleavage region that create a cleavage sequence for furin-like proteases at the R247 position (the multi-basic furin consensus sequence is indicated in bold letters). Numbers indicate the amino acid positions in the SA11 VP4 sequence.

chimeric rotaviruses will allow the generation of effective candidate vaccines for rotavirus diseases in the future.

The spike proteins of many enveloped viruses are initially synthesized as inactive precursors, which require proteolytic cleavage to yield fusion-competent forms, resulting in viral entry. Therefore, proteolytic cleavage of the precursor spike proteins at mono- or multi-basic cleavage site(s) by host proteases is an essential process for their conversion into an active state and for rendering the virus particles infectious (27). The mono-basic cleavage site and multi-basic cleavage site are recognized and cleaved by exogenous trypsin-like proteases and endogenous furin-like proteases, respectively. VP4, the spike protein of non-enveloped rotaviruses, resembles the precursor spike proteins of enveloped viruses (so-called class I fusion proteins) because they exhibit substantial structural and functional similarities (28). VP4 is cleaved at mono-basic cleavage sites by trypsin into VP8* and VP5* as active states, resulting in activation of rotavirus infectivity (29). To examine the possibility that changing of the VP4 trypsin cleavage site into a furin cleavage site might allow engineering of a rotavirus that can carry out multicycle replication without trypsin, we engineered and characterized a recombinant rotavirus expressing mutant VP4 that can be cleaved by furin-like protease as well as trypsin (9) (Fig. 3b). Unexpectedly, this VP4 cleavage site mutant virus could not carry out multicycle replication without an exogenous protease, although the mutant VP4s on virions were efficiently proteolysed by furin-like proteases. Because nascent virions containing already cleaved VP4s in cells were significantly constrained within the cells, it is conceivable that intracellular activation of VP4 by furin may be disadvantageous for rotavirus infectivity, possibly owing to an inefficient virus-release process. Therefore, in addition to an understanding of rotavirus biology regarding the linkage between VP4 cleavability and impaired virion release, future research on rotavirus reverse genetics should also include the development of live vaccines through the introduction of predetermined mutations for attenuation into defined genes.

Following our initial report of a rotavirus reverse genetics system, two other approaches based on this system were reported (Table 1). Troupin *et al.* (12) reported a method for generating a recombinant rotavirus containing a cDNA-derived rearranged NSP3 gene segment. This method is logically based on the observation that a rearranged NSP3 segment is preferentially packaged into progeny virions (30). By extensive serial passage of lysates of COS-7 cells transfected with a T7 plasmid encoding the rearranged NSP3 gene, and infected with rDIs-T7pol and a helper rotavirus, a recombinant rotavirus containing a cDNA-derived rearranged NSP3 gene segment was recovered. This strategy can theoretically be applied to other gene segments (VP6, NSP1–5) for which rearrangements

Table 1. Reverse genetics systems developed for members of the family *Reoviridae*

Genus	Species	No. gene segments	Helper virus-driven or helper virus-free	Year	Ref.
Rotavirus	Rotavirus	11	Helper virus-driven	2006	(7)
			Helper virus-driven	2010	(12)
			Helper virus-driven	2010	(10)
Orthoreovirus	Mammalian orthoreovirus	10	Helper virus-driven	1990	(38)
			Helper virus-free	2007	(32)
			Helper virus-driven	2008	(39)
			Helper virus-free	2010	(33)
Orbivirus	Bluetongue virus	10	Helper virus-free	2008	(34)
			Helper virus-driven	2010	(40)
	African horse sickness virus	10	Helper virus-driven	2010	(41)

have been detected (11). Furthermore, Trask *et al.* (10) reported a procedure for generating a recombinant rotavirus possessing a cDNA-derived NSP2 gene segment. The authors used a *ts* mutant of NSP2, with RNAi-mediated degradation of NSP2 mRNAs to select a recombinant rotavirus evading both selection mechanisms. A T7 plasmid encoding the NSP2 gene containing silent mutations to evade the RNAi degradation was transfected into COS-7 cells that had been infected with rDIs-T7pol. The transfected cells were then infected with the *ts* mutant as a helper virus. A recombinant rotavirus containing a cDNA-derived NSP2 gene segment was rescued by sequential passage of cell lysates at non-permissive temperature in MA104 cells expressing siRNA that degrades NSP2 mRNAs of the *ts* mutant. This method can theoretically be extended to other gene segments (VP1–4, VP6–7, NSP1–2, and NSP5) to which *ts* mutations have been mapped (31).

However, in spite of these advances, we must establish further selection systems for the other eight viral segments than the three (VP4, NSP2, and NSP3) for which reverse genetics systems have been developed.

INCREASED EFFICIENCY OF ROTAVIRUS REVERSE GENETICS SYSTEMS

Because the efficiency of virus recovery is almost entirely dependent on the selection mechanisms used against a background of helper viruses, several modifications to a selection protocol can be made that would increase the efficiency of recovery of recombinant viruses. A modified version of a reverse genetics system was described involving two independent selection mechanisms (10). As described above, the authors used a *ts* mutant and RNAi-mediated degradation to isolate a recombinant rotavirus that evades both selection mechanisms, resulting in enhanced rescue efficiency. For this method, the use of RNAi was less crucial but enhanced the recovery. We have also successfully combined the RNAi-based selection mechanism with the neutralizing antibody-based approach, resulting in significantly improved rescue of VP4 mutant viruses (S. Komoto and K. Taniguchi, unpubl. data, 2013), which may also be useful for the rescue of a viral segment encoding another virion surface protein (i.e. VP7 gene). Thus, since the initial report of a reverse genetics system, the efficiency of virus recovery has been improved.

APPROACHES FOR A HELPER VIRUS-FREE REVERSE GENETICS SYSTEM FOR ROTAVIRUSES

Although the current reverse genetics systems led to the recovery of recombinant rotaviruses containing a cDNA-derived gene segment, thus contributing significantly to our knowledge of these viruses, there are clear limitations to these systems owing to the requirement of strong selection mechanisms. So far, systems have been described for only three of the 11 viral segments. Alternatively, a helper virus-free reverse genetics system may need to be developed to generate an infectious rotavirus entirely from cDNAs, as has been done for other members of the *Reoviridae*, mammalian orthoreovirus (reovirus) (32, 33) and bluetongue virus (34), each possessing a genome of 10 segments (Table 1). Recent advances in helper virus-free reverse genetics systems strongly suggest that it is possible to initiate the replication cycle of *Reoviridae* viruses entirely from cDNAs. These helper virus-free systems bypass the early virus replication stages by introducing a set of positive-strand RNAs encoding a viral genome into cells; that is, 10 transcripts corresponding to native viral mRNAs serve as templates for translation and dsRNA replication, and ultimately lead to the formation of infectious virus particles.

Reovirus was the first member of the *Reoviridae* for which a helper virus-free reverse genetics system was established. The rescue of infectious reovirus from cloned cDNAs was initially established for serotype 3 prototype strain, type 3 Dearing (T3D) (32). Subsequently, a similar approach was used to recover serotype 1 prototype strain, type 1 Lang (T1L) (33). The initial helper virus-free reovirus reverse genetics system consists of 10 plasmids, each containing a full-length cDNA copy of a single reovirus gene segment flanked by T7 RNA polymerase promoter and HDV ribozyme sequences. These T7 plasmids are presumed to generate 10 transcripts corresponding to native reovirus mRNAs. The reovirus replication cycle can be launched by transfection of the 10-plasmid set encoding a genome of reovirus into cells expressing T7 RNA polymerase. However, there have been no reports of this approach being used successfully to generate rotavirus or bluetongue virus.

A helper virus-free reverse genetics system for bluetongue virus is logically based on the observation that the 10 transcripts of bluetongue virus produced *in vitro* from purified native viral cores can initiate the replication cycle of this virus by introducing them into cells (35). This phenomenon was then developed into a helper virus-free reverse genetics system through the engineering of the 10 plasmids encoding the genome of bluetongue virus type 1 (BTV-1) (34). Like reverse genetics systems for rotavirus and reovirus, this approach also involves T7 RNA polymerase to generate the authentic 5′ terminus of each positive-strand RNA from cDNA, but uses an *in vitro* run-off transcription mechanism to generate the native 3′ terminus from a linearized plasmid. The replication cycle of bluetongue virus can be initiated by transfection of the 10 T7 transcript set encoding the genome of bluetongue virus into cells. However, there have been no reports of this approach being used successfully to generate rotavirus or reovirus.

Despite extensive attempts, the helper virus-free approaches for reovirus and bluetongue virus described above have been applied to rotavirus without success so far. In our laboratory, we could readily generate infectious reovirus using a reovirus 10-plasmid set provided by Dr Dermody (Vanderbilt University, Nashville, TN, USA) by transfecting them into cells expressing T7 RNA polymerase (Komoto *et al.*, in prep.). Therefore, we recognize that the reagents and techniques used for rotavirus in our laboratory should not be the cause of the failure in the development of a helper virus-free reverse genetics system for rotavirus. Rather, several factors can be considered for the failure: (i) rotavirus has one more gene segment than reovirus; (ii) rotavirus can replicate to the lower virus titers (10^8 PFU/mL) than can reovirus (10^9 PFU/mL); (iii) cultivation of rotavirus strictly requires removal of FCS and addition of trypsin, which damages cells that have been infected with rDIs-T7pol and transfected with a large quantity of plasmid DNAs; and (iv) the number of virus particles per infectious unit is much higher for rotavirus (36). To circumvent the potential involvement of some of these issues, we have carried out modified experiments in various aspects for the development of a helper virus-free system using the

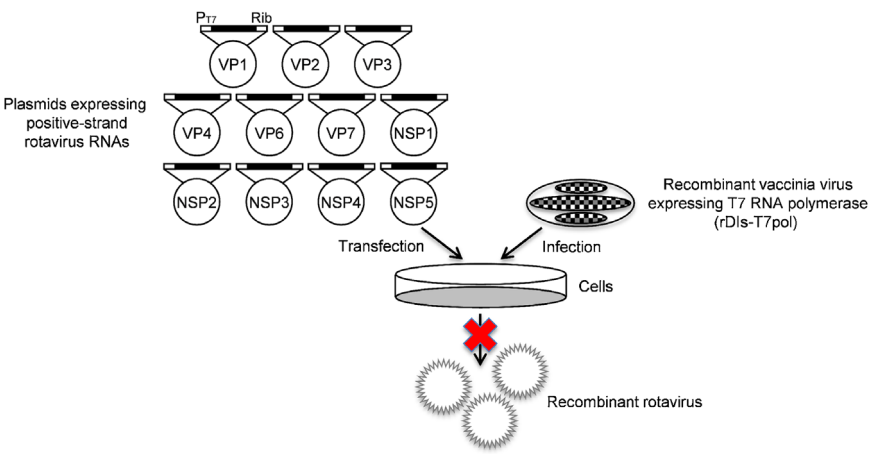

Fig. 4. Experimental strategy for developing a helper virus-free reverse genetics system for rotavirus. The 11-plasmid set encoding the genome of rotavirus is transfected into cells that have been infected with recombinant vaccinia expressing T7 RNA polymerase (rDIs-T7pol). Each of the 11 rotavirus plasmids contains a full-length rotavirus gene segment cDNA, flanked by T7 RNA polymerase promoter and HDV ribozyme sequences. In transfected cells, T7 RNA polymerase catalyzes the expression of 11 transcripts corresponding to the native rotavirus mRNAs from the 11 rotavirus plasmids. However, experiments involving modified versions of this strategy have not led to the successful generation of an infectious rotavirus entirely from cDNAs.

11-plasmid set encoding the genome of rotavirus (4). We have also tried a repeated transfection procedure involving a rotavirus 11-plasmid set with intervals of several hours or once a day in cells expressing T7 RNA polymerase (S. Komoto and K. Taniguchi, unpubl. data, 2013) because this protocol improved the low efficiency of generation of iPS cells with the plasmid transfection method (37). However, experiments involving these approaches have not led to success so far (Fig. 4). Further studies regarding rotavirus replication involving the forward genetics approach and current reverse genetics systems will be needed for the development of a helper virus-free reverse genetics system for rotavirus.

CONCLUSION

Reverse genetics technology has had a significant impact on our understanding of virus replication and pathogenesis, as well as on the development of vaccines and viral vectors. Also, in the field of rotavirus, current helper virus-driven reverse genetics systems are contributing to our understanding of this medically important virus, which is difficult to investigate using conventional virological methods. However, we are still eagerly awaiting the next breakthrough, for example, the development of a helper virus-free reverse genetics system for generating an infectious rotavirus entirely from cDNAs, as has been achieved for other members of the *Reoviridae* family.

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DISCLOSURE

The authors have no conflicts of interest to disclose.

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