COMMENTARY

Reverse genetics of rotavirus

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The genetics of viruses are determined by mutations of their nucleic acid. Mutations can occur spontaneously or be produced by physical or chemical means: for example, the application of different temperatures or mutagens (such as hydroxylamine, nitrous acid, or alkylating agents) that alter the nucleic acid. The classic way to study virus mutants is to identify a change in phenotype compared with the wild-type and to correlate this with the mutant genotype ("forward genetics"). Mutants can be studied by complementation, recombination, or reassortment analyses. These approaches, although very useful, are cumbersome and prone to problems by often finding several mutations in a genome that are difficult to correlate with a change in phenotype. With the availability of the nucleotide sequences of most viral genomes and of the tools of genetic engineering, this stratagem has drastically changed. One can now start with rationally engineering particular mutations in individual viral genes, followed by production of infectious viral particles and exploration of the phenotype ("reverse genetics"). Whereas forward genetics investigates the genetics underlying a phenotype, reverse genetics observes the phenotypic changes arising from genetic changes that were "made to order."

Reverse genetics is a relatively straightforward task with DNA viruses because virtually all viral DNA genomes, which can be mutated in vitro, are infectious upon transfection. Reverse genetics of RNA viruses involves the manipulation of their genomes at the cDNA level, followed by procedures to produce live infectious progeny virus (wild-type or mutated) after transfection of cDNAs into cells. To achieve this end, co- or superinfection with a helper virus has been used in initial attempts. However, because virus particles carrying the engineered genome may be very difficult to separate from helper virus, the final aim is to create helper virus-free, plasmid-only– or RNA-only–based systems. A tractable, helper virus-free reverse genetics system is a powerful tool, because it allows precise assignment of phenotypic changes to engineered mutations in comparison with the wild-type phenotype and genome. Reverse genetics techniques can help clarify structure/function relationships of viral genes and their protein products and also elucidate complex phenotypes, such as host restriction, pathogenicity, and immunogenicity.

Kanai et al. (1) have now developed a reverse genetics system for rotaviruses (RVs), which are a major cause of acute gastroenteritis in infants and young children and in many mammalian and avian species. Worldwide RV-associated disease still leads to the death of over 200,000 children of <5 y of age per annum (2) and thus represents a major public health problem.

The work by Kanai et al. (1) is the most recent addition to a long list of plasmid-only–based reverse genetics systems of RNA viruses, a selection of which is presented in Table 1 (3–13). A potent reverse genetics system for RVs (1) represents a long-awaited breakthrough and is a major technological advance over the most-sophisticated helper virus-dependent reverse genetics procedures recently developed for RVs (14, 15).

Using a previously validated approach (12), Kanai et al. (1) constructed plasmids, each containing the cDNA of 1 of the 11 RV RNA segments (SA11 strain), inserted between a T7 RNA polymerase (T7Pol) promoter (5′end) and the antigenomic hepatitis δ-virus ribozyme (3′end), from which, upon cotransfection into BHK cells constitutively expressing T7Pol, authentic full-length viral ss(+)RNA transcripts would be synthesized. Although this procedure or a modification using ss(+)RNAs transcribed from cDNA clones in vitro was successful in "rescuing" infectious reovirus (12) or bluetongue virus (13) particles, it did not lead to the recovery of infectious RV progeny (1, 16). Based on previous findings that fusion-associated small transmembrane (FAST) proteins (encoded by *Aquareovirus* and some *Orthoreovirus* species) increased the yield of heterologous mammalian orthoreovirus substantially (1, 17), RV replication was found to be significantly increased in infected cells when a reovirus FAST protein was expressed from a transfected plasmid (1). Furthermore, the overexpression of the vaccinia virus-capping enzyme increased translatability of reovirus (+)ssRNAs, and coexpression of both greatly

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Table 1. Plasmid-only based reverse genetics systems of selected RNA viruses

Virus	Genomic RNA	Virus family	Lead publication
Poliovirus	ss(+)RNA	*Picornaviridae*	Racaniello and Baltimore (3)
Astrovirus	ss(+)RNA	*Astroviridae*	Geigenmueller et al. (4)
Mouse norovirus	ss(+)RNA	*Caliciviridae*	Chaudhry et al. (5)
HIV	ss(+)RNA dim rev tr	*Retroviridae*	Sadaie et al. (6)
HCV	ss(+)RNA	*Flaviviridae*	Wakita et al. (7)
Influenza A virus	ss(−)RNA 8 seg	*Orthomyxoviridae*	Fodor et al. (8); Neumann et al. (9)
Bunyavirus	ss(−/+)RNA 3 seg	*Bunyaviridae*	Bridgen and Elliott (10)
IBDV	dsRNA 2 seg	*Birnaviridae*	Mundt and Vakharia (11)
Reovirus	dsRNA 10 seg	*Reoviridae*	Kobayashi et al. (12)
BTV	dsRNA 10 seg	*Reoviridae*	Boyce et al. (13)
Rotavirus	dsRNA 11 seg	*Reoviridae*	Kanai et al. (1)

A more comprehensive list (up to 2005) is presented in table S1 of Kobayashi et al. (12). BTV, bluetongue virus; dim rev tr, dimeric reverse transcribing; ds, double stranded; HCV, hepatitis C virus; HIV, human immunodeficiency virus; IBDV, infectious bursal disease virus; seg, number of segments; ss, single-stranded; +/−, of positive or negative sense.

improved mammalian orthoreovirus rescue in established reverse genetics systems (1, 12). Following these leads, the 11 RV cDNA-containing plasmids were cotransfected into cells constitutively expressing T7Pol (BHK-T7) together with plasmids expressing a reovirus FAST protein and the two subunits of the vaccinia virus-capping enzyme. Following passage of lysates of transfected cells in MA104 cells, infectious RV was recovered (1). The introduction of genetic markers (restriction enzyme recognition sites) into the cDNAs of several RV genes confirmed that the RV rescue was genuine and not due to contamination (1). The rescued RVs had replication kinetics and peak titers that were indistinguishable from those of the native parent virus.

This reverse genetics system permitted the recovery of RVs with genes reassorted "to order," as demonstrated with one example as proof-of-principle (1). Furthermore, the system was able to explore the functions of a RV-encoded protein. A deletion mutant truncated of the nucleotides encoding the 108 C-terminal amino acids of RV NSP1 (nonstructural protein 1) was created by reverse genetics: as expected, the phenotype revealed a decrease of replication in different cell lines, and the molecular mechanisms were elucidated by experiments probing for components of the intracellular pathway with which NSP1 interacts (1). Moreover, using the split GFP system for fluorescent signaling (18), one of the two GFP subunit genes was fused to the RV NSP1 gene (downstream of nucleotides encoding the C terminus of the NSP1 ORF), and the other one was expressed from a transfected plasmid; viable recombinant RV was rescued, and both GFP subunits were functionally active by complementing each other. Based on previous findings of the nonessential role of NSP1 for RV replication in vitro, a construct was made expressing a foreign gene (encoding a luciferase) from an NSP1-based fusion gene: a rescued RV recombinant carrying this transduced gene produced a bioluminescence signal and was shown to be useful for dose-dependent screening of antivirals (here: ribavirin) (1).

The plasmid-only–based reverse genetics system for RVs is thoroughly convincing and very versatile. There will be many applications, and questions of the basic molecular biology of RVs can be addressed, which include the identification of the packaging signals of the RV RNAs, the discovery of sequences of individual RNA segments determining the assortment process, and the exploration of the components of complex biological phenotypes, such as host-range restriction, virulence, and attenuation. Rotaviruses prominently exposing highly cross-reactive epitopes (inducing cross-protective immunity) may lead to a universal rotavirus vaccine.

As a rule, reverse genetics systems of RNA viruses have been substantially improved after their first description: for example, by locating the cDNAs of several or all RNA segments on one plasmid, thus reducing the number of plasmids to be cotransfected (19, 20). Some of the difficulties and challenges, but also the enormous potential of effective reverse genetics systems for different RNA viruses, have been reviewed recently (21).

The work by Kanai et al. (1) represents a key achievement in RV research; it is very exciting and will move the field forward in many laboratories as reverse genetics systems for many other RNA viruses (influenza virus, bunyavirus, measles virus, lyssa virus, poliovirus, reovirus... to name a few) have done.

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