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Commentary

Reverse genetics of negative-strand RNA viruses: Closing the circle

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The study of viruses and their interactions with host cells and organisms has benefited greatly from the ability to engineer specific mutations into viral genomes, a technique known as reverse genetics. Genome manipulations of DNA viruses, either by transfecting cells with plasmids encoding the viral genome (1) or by heterologous recombination of plasmids bearing viral sequences with the virus genome (2–4), were the first to be performed. Positive-strand RNA virus genome manipulation followed quickly, partly because the viral genome is also mRNA sense. Simply transfecting plasmids, or RNA transcribed from plasmids, containing the poliovirus genome into susceptible cells resulted in the recovery of infectious poliovirus (5, 6). The negative-strand RNA viruses include a number of human and animal pathogens such as influenza A, B, and C viruses, hantaviruses, Lassa virus, rabies virus, Ebola virus, Marburg virus, measles virus, canine distemper virus, rinderpest virus, respiratory syncytial virus, mumps virus, human parainfluenza virus types 1–4, and Nipah virus (which recently emerged in Malaysia, causing respiratory distress and encephalitis in pigs and humans). However, the genomes of the negative-strand RNA viruses have been less amenable to artificial manipulation for several reasons: (*i*) precise 5′ and 3′ ends are required for replication and packaging of the genomic RNA; (*ii*) the viral RNA polymerase is essential for transcribing both mRNA and complementary, positive-sense antigenome template RNA; and (*iii*) both genomic and antigenomic RNAs exist as viral ribonucleoprotein (RNP) complexes (reviewed in ref. 7). The segmented genomes of influenza viruses, bunyaviruses, and arenaviruses allowed some genetic manipulation through the isolation of reassortant viruses, but manipulation of the complete genome of segmented negative-strand RNA viruses has progressed slowly, hampered by the very fact that the genome is segmented.

In this issue of the *Proceedings*, Neumann and coworkers (8) have come full circle on recovering recombinant, segmented negative-strand RNA viruses with the production of influenza virus entirely from plasmid DNA and driven only by the host cell transcription and translation machinery. Coming nearly 10 years after the first published reports of influenza virus genome manipulation (9) and after another *Proceedings* article describing the generation of recombinant bunyaviruses wholly from cDNA by using a recombinant vaccinia virus-driven system (10), virologists finally have acquired the tools necessary to perform sophisticated and comprehensive investigations of the role of all influenza virus proteins and RNA elements in replication and pathogenesis.

The influenza virus RNPs, upon their release into the cytoplasm of an infected cell, enter the cell nucleus, and the influenza virus polymerase complex, consisting of the PA, PB1, and PB2 proteins, begins to transcribe the genomic RNA into mRNA and a positive-sense antigenome RNA that serves as the template for the production of genome RNA. Although influenza virus was the first negative-strand RNA virus to have individual virus genes replaced by artificially manipulated segments, the difficulty in dealing with a segmented RNA genome, as well as the use of labor-intensive and selection-dependent techniques to drive reverse genetics has hindered the application of this technology. Nonetheless, many important discoveries pertaining to individual influenza virus proteins as well as demonstrating the use of influenza virus to serve as a viral expression vector have been obtained by application of the existing reverse genetics technology (reviewed in refs. 7 and 11).

Neumann and coworkers (8) have established a system that conscripts the host cell into making the equivalent of newly released RNPs by cotransfecting eight plasmids encoding each of the influenza virus genomic RNA segments under control of the RNA polymerase type I (pol I) promoter and transcription terminator along with four plasmids encoding the polymerase complex proteins and nucleoprotein (NP) cDNAs under control of an RNA polymerase type II (pol II) promoter. Although the concept of cotransfecting multiple plasmids to reconstitute a biochemical activity was pioneered for studying herpes virus DNA replication (12), the daunting nature of this 12–17 plasmid transfection (a likely record for most plasmids transfected into one cell) still results in approximately 1 in 1,000 cells producing infectious virus. The lack of a helper influenza virus allows the virus from the initial transfection to be characterized immediately, thus limiting the chance of viruses containing reversions or second-site mutations from becoming significant contaminants. One can only speculate as to how quickly our knowledge of influenza virus will progress, now that every nucleotide of the viral genome can be mutated and engineered back into the genome, in nearly endless combinations with other mutations.

As with most important scientific advances, the work of Neumann and coworkers builds on a large body of experiments that have identified the basic requirements for replicating and packaging influenza virus RNA segments. The technique used first to introduce a new, artificial RNA segment into influenza virus (13) and refined subsequently to create influenza viruses containing neuraminidase (NA) proteins derived from plasmid cDNAs (9) relied on reconstitution of viral RNPs from *in vitro*-transcribed RNA and purified nucleocapsid proteins (Fig. 1). The protein-RNA complex was transfected into cells, followed by infection with a helper influenza virus. The application of a selection pressure against the helper virus facilitates the detection of progeny virus containing the plasmid DNA-derived RNA segment. Although a tour de force of molecular biology at the time, the technique requires the purification of large amounts of viral nucleocapsid proteins and is most efficient when a strong selection pressure can be applied against the helper virus.

The use of pol I transcripts to produce artificial influenza virus RNA segments was pioneered by Hobom and colleagues (14–16). Unlike the mRNA transcripts produced by pol II, the primary RNA transcripts synthesized by pol I are ribosomal RNAs that possess neither a 5′ cap structure nor a 3′ poly(A) tail. Zobel and coworkers (16) successfully produced artificial influenza virus RNA segments with precise 5′ and 3′ ends, and

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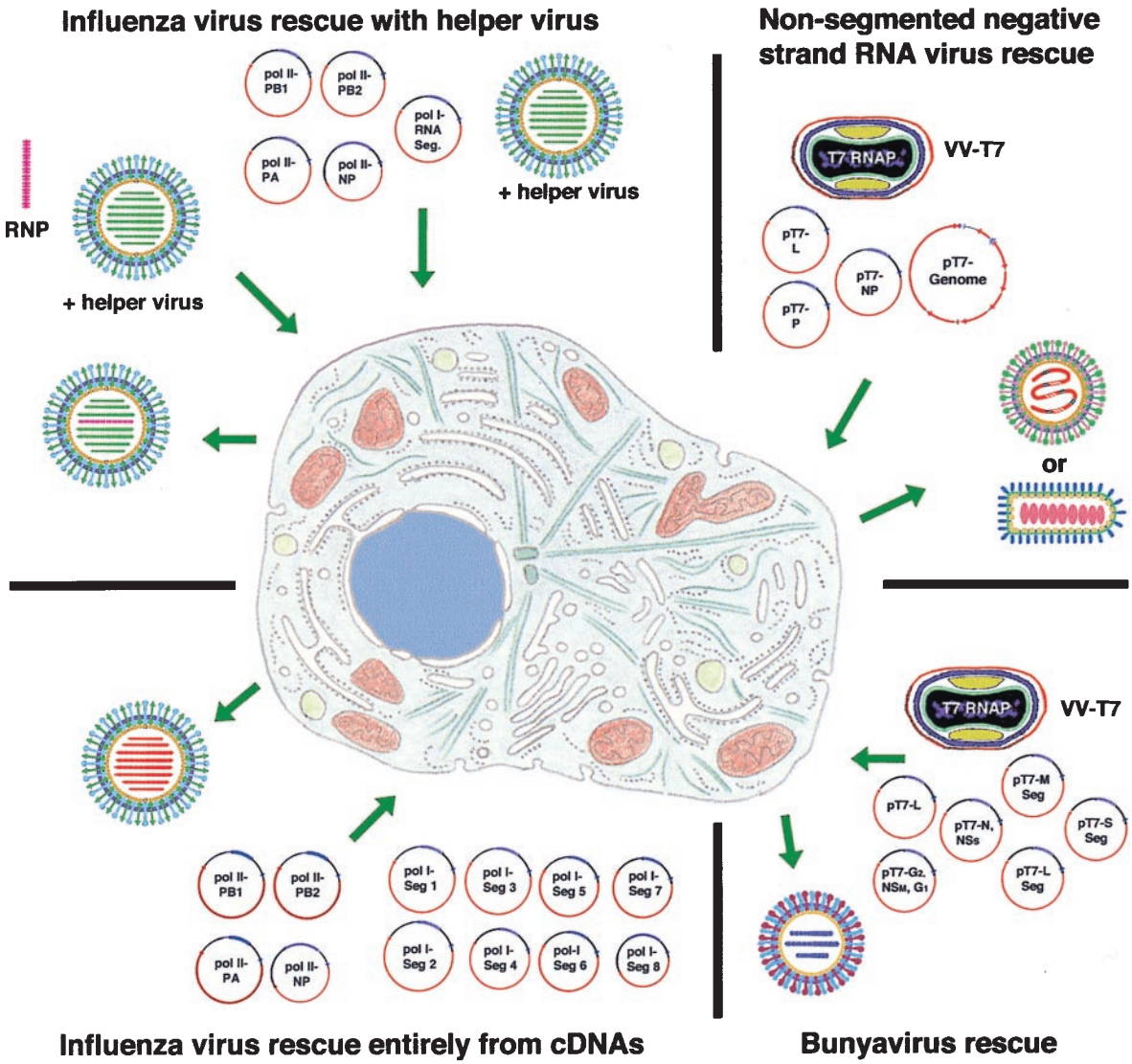

FIG. 1. Methods used to recover negative-strand RNA viruses from plasmid DNA. Several methodologies have been used to rescue negative-strand RNA viruses from plasmid-derived DNA. The initial replacement of individual RNA segments of influenza virus involved either (*i*) the *in vitro* reconstitution of RNPs or (*ii*) the *in vivo* assembly of RNPs after transfection of a cell with plasmids that use pol II promoters driving the expression of the PA, PB1, PB2, and NP proteins, and pol I promoters and terminators controlling viral genome synthesis. In either case, transfection was followed by infection with a helper influenza virus. Nonsegmented negative-strand virus rescue involves the transfection of plasmids encoding the viral P, N, and L proteins (and sometimes other viral proteins depending on the virus), as well as the viral antigenome, all under control of the bacteriophage T7 RNAP promoter. The T7 RNAP is provided by either infection with vv-T7, a recombinant vaccinia virus that expresses T7 RNAP or by transfecting into cell lines that stably express the protein. Bunyavirus rescue requires the transfection of plasmids encoding the three RNA segments in the antigenomic sense, along with three plasmids encoding the viral proteins, all under control of the T7 RNAP promoter. Influenza virus rescue entirely from plasmid DNA involves the transfection of plasmids encoding each of the eight RNA segments (under control of the pol I promoter and terminator) and plasmids encoding the four proteins that make up the polymerase complex (under control of the pol II promoter).

subsequent studies demonstrated that these genomic RNA constructs could be recognized and replicated by the influenza virus polymerase complex and packaged into progeny influenza viruses (14, 15). Pleschka and coworkers (17) used this technique to replace the viral RNA segment encoding the NA glycoprotein with a plasmid-based construct, showing the technique could substitute for RNP reconstitution in replacing single viral RNA segments. In addition, the artificial pol I transcript could be replicated and packaged into RNPs simply by cotransfecting plasmids encoding the PA, PB1, PB2, and NP proteins (17), shown previously to be the minimal proteins required to reconstitute influenza virus polymerase activity (18). The system described by Neumann and coworkers (8) represents the logical and important culmination of this body of work, finally resulting in the ability to manipulate every gene in the influenza virus genome.

Concurrent with efforts to perform reverse genetics with influenza virus, techniques to manipulate the genomes of nonsegmented negative-strand RNA viruses were being developed (reviewed in ref. 19). The task proved quite frustrating until Schnell and coworkers (20) made the somewhat counterintuitive, yet innovative, discovery that cotransfecting plasmids encoding the rabies virus L, P, and N protein cDNAs, as well as the viral antigenome, under control of the bacteriophage T7 RNA polymerase (T7 RNAP) promoter, into cells infected with a recombinant vaccinia virus expressing the bacteriophage T7 RNAP protein (vv-T7) (21) resulted in the recovery of recombinant rabies virus. The reverse genetics technique was quickly adapted by laboratories studying other nonsegmented negative-strand RNA viruses, resulting in the rescue of vesicular stomatitis virus (22, 23), measles virus (24), respiratory syncytial virus (25), Sendai virus (26, 27), rinder-

pest virus (28), human parainfluenza virus 3 (29, 30), simian virus 5 (31), and Newcastle disease virus (32). In some of these studies, rescue also has been accomplished by using the genome sense RNA (27, 30). Some refinements to the original technique have been made, such as the use of stably transfected cell lines expressing the T7 RNAP (in lieu of vv-T7 infection), or one or more of the viral proteins required for genome replication (24).

Several technical aspects of plasmid-based rescue of influenza virus as described by Neumann and coworkers (8) should be explored further. The number of recombinant viruses rescued can be increased nearly 10-fold by including plasmids encoding the hemagglutinin, NA, M_1, M_2, and NS_2 proteins under control of the pol II promoter in the transfection. The simplest explanation for this would be that the presence of the other influenza virus structural proteins allows packaging of RNPs at an earlier time posttransfection, resulting in more progeny virus being released. Alternatively, the M_1 and NS_2 (suggested to be renamed the nuclear export protein, NEP) proteins are known to function in modulating the import and export of RNPs from the nucleus of influenza virus-infected cells (33, 34), and the presence of these proteins alone may increase the amount of cytoplasmic RNPs available for packaging into progeny virions. Also, the production of spliced mRNAs from RNA segments 7 and 8 depends only on host cell factors, and the relative amounts of the spliced mRNAs present vary from cell to cell type (35, 36), which may affect virus recovery. The major difference between plasmid-based rescue of most nonsegmented negative-strand RNA viruses and influenza viruses involves the use of plasmids expressing antigenome or genome-sense RNA transcripts, respectively. Although the virus rescue efficiency of the plasmid-derived influenza virus is quite good, the use of an antigenomic plasmid may increase efficiency even more. The application of pol I-mediated expression of nonsegmented and other segmented negative-strand RNA virus genomes has yet to be explored but the success of Neumann and coworkers will no doubt result in a flurry of activity. However, although influenza virus has evolved to replicate in the nucleus and to exploit the cell-splicing machinery, for other RNA viruses that replicate in the cytoplasm, successful use of the pol I recovery system will depend on the absence of cryptic splicing signals in RNA transcripts.

Plasmid-based recovery of influenza virus allows investigation of aspects of the influenza virus life cycle that are known to involve multiple RNA segments, such as the neurovirulence of influenza A/WSN/33 in mice (reviewed in ref. 37) or viral polymerase functions. The engineering of influenza virus vaccines also should be improved quickly, because nucleotide changes correlating with attenuating or temperature-sensitive phenotypes now can be specifically identified and introduced in various combinations to produce new potential vaccines. If a properly attenuated genetic background can be constructed independent of the hemagglutinin and NA genes, the time needed to generate new vaccine viruses should be reduced to days rather than weeks. The ability of influenza virus to package additional RNA segments also may allow the virus to be used as a vector for delivering proteins to cells for therapeutic purposes, although the stability of these viruses needs to be investigated further.

Along with technological advances such as the ability to recover "designer" viruses, the relative ease of constructing influenza virus genes from synthetic oligonucleotides (38) and the sequencing of genes from "extinct" influenza virus strains such as the highly virulent 1918 Spanish influenza virus strain (39), comes a responsibility to avoid the construction of viruses that may pose a public health hazard. Restraint should be practiced, especially when dealing with factors associated with increased virulence, such as hemagglutinin proteins with multibasic cleavage sites (reviewed in ref. 40), or in the use of NA subtypes known to confer trypsin-independent cleavage of hemagglutinin (41).

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